DIETARY FACTORS AFFECTING BLOOD SPOT INCIDENCE AND CHANGES IN THE VASCULAR SYSTEM OF THE HEN

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One of the oldest problems of concern to the egg-producing industry is the occurrence of blood spots in eggs. While many dietary factors have been reported to affect the incidence of eggs containing blood spots, these relationships have not established the direct cause of this abnormality.

Studies were conducted to determine the relationship of the vascular integrity of birds to the percent of blood-spot eggs they produce. Both a normal commercial strain and an experimental strain producing a high incidence of blood-spot eggs were examined.

Four feeding trials were conducted in which the progeny from hens of both strains treated with a copper-deficient diet were subjected to the stress of β-aminopropionitrile (BAPN) toxicity. Production, fertility, hatchability and liver copper levels were used to determine the

effect of the maternal copper-deficient diet. Striking differences were detected between the response of the two strains to this dietary treatment. Egg production of the normal strain was markedly affected, decreasing from 80% to less than 6% in 5 days. During the same period, production in the blood-spot strain showed only a slight decrease. The level of copper in the livers of the normal hens was reduced to a significantly lower level than in the blood-spot strain by the dietary treatment. No differences were detected between the response of chicks from the two strains of hens to the BAPN toxicity. The incidence of death, aortic rupture, severity of leg disorders and growth depression in the 2 strains was not statistically different. Electron micrographs of the aortas from day-old chicks treated with the parental low copper diet indicated similar disruptions in the vascular wall of both strains.

Lack of integrity in the follicular vascular system in the blood-spot strain would not be the only abnormality which could account for the high incidence of blood spots produced. The critical period in the formation of blood spots appears to be during the rapid phase of ova growth. The rate of ova growth during the rapid phase and the ultimate size of the mature ova were compared in the 2 strains. No differences between the strains were detected in the rate of rapid growth. However, it was found that the size

of the mature ova was significantly smaller in the blood-spot strain.

Blood pressure is often mentioned as a possible factor influencing the occurrence of blood spots. The systolic blood pressure in hens of the blood-spot strain was significantly altered by feeding tapazole, a goitrogenic agent. While the systolic pressure was significantly decreased and ultimately increased from the normal pretreatment pressure, no significant change in the incidence of blood spots was observed.

A final study was conducted in which the physical state of follicles removed from both strains was compared. Of the characteristics compared, the high incidence of blood clots in the follicle wass and wide stigmas in the blood-spot strain are most noteworthy. The relationship of these characteristics to the high incidence of blood spots has not been established at this time.

INTRODUCTION

One of the oldest problems of concern to the egg-producing industry is the occurrence of blood spots in eggs. Today, with the development of each new strain of high-producing hens, this problem becomes more costly in a highly competitive industry.

While the incidence of blood spots can be minimized by proper management and the feeding of correctly-balanced diets, there has been no means discovered to eliminate or entirely prevent their formation.

Since the first studies in the early 1940's, very few new approaches in attempting to solve this problem have been reported. Many factors which apparently intensify or alleviate the abnormal conditions associated with the formation of blood spots have been published but no single factor or condition directly responsible for their formation has been reported. It is the commonly held theory today that blood spots result from a ruptured blood vessel in the follicle wall. This rupture and the accompanying loss of blood may occur either at the time of ovulation or prior to ovulation during the rapid phase of ova growth. No hypothesis can be found in the literature, however, explaining the basic cause for the occurrence of these ruptures in the first place.

This lack of knowledge concerning the reasons for the occurrence of blood spots is evident when one realizes that no means has been found of reducing the incidence below the normally accepted range of from 2 to 6%.

It was the purpose of this research to not only study new approaches in an effort to discover the cause of preovulatory intrafollicular hemorrhage, but to reinvestigate some of the commonly accepted ideas already developed.

CHAPTER I

LITERATURE REVIEW

Since a large percentage of blood-spot eggs are classed as inedible and require special handling, they constitute a substantial financial loss to the poultry industry each year. With the innovation of automatic blood spot detectors, producers eliminate many perfectly sound eggs rather than risk the occurrence of a blood-spot egg among those sent to fresh market. The importance of blood spots as a factor in consumer demand was pointed out by Stadelman (1950). Results of a survey showed that 7% of all consumer complaints about egg quality concerned blood and meat spots which found their way into market channels. The presence of detectable amounts of blood in eggs may cause a consumer to stop using eggs until they recover from this adverse psychological reaction.

The oldest reference, often cited, referring to the presence of blood spots in eggs dates back to around 300 B.C. when Aristotle explained the condition as being due to a premature expulsion of the yolk. Some of the first and most extensive studies on the blood-spot problem were conducted at the University of Illinois by Nalbandov and Card (1944). These authors reached the conclusion that

blood spots were the result of a rupturing of blood vessels in the follicle wall sometime prior to ovulation and during the active growing stage of the follicle. They state that if bleeding is significant in amount, the resulting clot will adhere to the vitelline membrane and will remain attached to the yolk while the latter passes through the oviduct. If this bleeding is extensive, the blood will accumulate in the hollow stalk of the follicle and the clot appearing in the laid egg will not be attached to the yolk but floating free in the albumen.

Jeffrey (1945) made an extensive study of the origin of blood and meat spots using a different approach from those physiological methods usually employed. By recording the color and size of blood spots in each clutch position, the author determined the time of blood spot formation. His results in regard to the time of ovarian hemorrhages, during formation of the yolk, support the conclusions reached by other researchers.

Stiles et al. (1958), using ^{32}P-labeled red blood cells, developed a procedure for labeling blood spots in eggs from hens. If labeled cells were injected into a bird at known times during the ovulatory cycle, the time of subsequent blood spot formation could be correlated to this cycle. Blood spots formed after the labeled cells had been injected showed detectable radioactivity if the spots

contained more than 0.024 ml of blood. Although the authors claim that the time of blood spot formation can be determined by this method, they report very few results and state only that blood spot formation occurs within a 9-hour period prior to ovulation.

After the theory of blood spot formation in eggs was developed , various attempts were made to control the intrafollicular bleeding which is essential to their formation. Possible causes of the bleeding include abnormally slow blood-clotting time, abnormally weak capillary walls, a vitamin deficiency which would result in either of the first two and, finally, abnormally high blood pressure which would lead to frequent rupture of blood vessels.

Halman and Day (1935) found no correlation between productivity and the occurrence of egg faults and inferred that the condition had a hereditary basis. Quinn and Godfrey (1940) report that they found no significant correlation between percentage of blood spots and egg production, egg weight or body weight. They state, however, that a statistical analysis indicated significant breed and family differences in both yolk and albumen spots in eggs of Rhode Island Red, Wyandotte, White Leghorn and F_1 crosses of these breeds. Jeffrey and Pino (1943) concurred in the opinion that the condition was heritable. Additionally, they found that hens kept in laying cages produced 6.2% fewer blood spots

than their sisters maintained on the floor. They also tested the effect of systematic scaring of the flock and found that this did not increase the frequency of blood spots produced. No explanation was offered as to why reduced exercise from confinement reduced the incidence of blood spots while violent exercise did not increase the incidence. The importance of heredity in the production of blood spots was illustrated by Lerner et al. (1951) who estimated the heritability of the characteristic to be 0.5 or equal to all other factors combined. Sauter et al. (1952) reported that on the basis of strain differences within the New Hampshire breed, heredity appeared to be important in blood spot incidence. Breed difference tended to confirm this. The intensity and persistency of lay were reported to have little effect on the incidence of blood spots.

Seasonal variation in blood spot incidence has been reported. Lerner and Smith (1942) found significant seasonal differences with a definite increase after the first of April. Jeffrey (1945) reported the tendency to be highest at the beginning of the laying year and to decrease through the following August. Lerner and Taylor (1947) found an increase in incidence of blood spots until June followed by a subsequent decrease through September. A similar, though not identical, trend was reported by Sharma (1949). Denton (1947), using 3 separate periods

during the year, could find no seasonal influence on the presence of blood spots. Nalbandov and Card (1944) found a definite increase in blood spots from December through July. Sauter et al. (1952) reported there is a definite and statistically significant seasonal variation in incidence. Percentages ranged from 3.3 to 10.3 for White Leghorns and from 13.7 to 39.2 for New Hampshires. Percentages of total eggs having blood spots were lowest at the start of observations in December.

Stiles and Dawson (1959) found a higher incidence of blood spots in the first egg of a clutch than in succeeding clutch positions. The average weight of eggs containing blood spots was found to be significantly greater than the average weight of normal eggs when comparisons were made between eggs of the first clutch position and when all the eggs were compared disregarding clutch position. This difference was approximately 1.5 g. These authors further reported there was no correlation between the barometric pressure or net pressure changes for the area and the incidence of blood spots in eggs laid 24 to 48 hours later. They also compared normal eggs to eggs containing blood spots to determine if experiences existed regarding the time of oviposition, position in clutch and egg weight. In comparing normal and blood-spot eggs of a similar clutch

position, it was determined that the average oviposition time of blood-spot eggs occurred approximately 30 to 50 minutes sooner than normal eggs.

The effect of nutrition upon this defect has been investigated by many. Nalbandov and Card (1944, 1947) reported that the feeding of dehydrated, unjointed, young cereal grasses and permitting birds access to range substantially reduced the number of blood spots. Both the size and number of blood spots diminished rapidly and steadily when hens were turned on range. The authors stated that vitamins A, C, D, E, K and P supplements, administered singly, had no effect. Dehydrated alfalfa and grass meals are accepted components of poultry feeds. In addition to supplying nutrients, they serve as an economical means of providing dietary pigments for desired yolk color. The effects of dehydrated alfalfa on blood spot incidence are controversial. Sauter et al. (1952) reported that birds fed a ration containing 10% alfalfa consistently produced a lower percentage of both total blood spots and of medium and large blood spots. The average was 0.6% lower with Leghorns and 5.9% lower with New Hampshires. Carver and Henderson (1948) reported that hens fed high alfalfa levels produced fewer blood-spot eggs than those fed little or none. Bearse (1955) stated that suboptimal levels of vitamin A caused more and above optimal levels caused fewer

blood spots than did the level recommended by the National Research Council (2000 U.S.P. units/lb). This does not agree with the earlier conclusions of Nalbandov and Card (1944, 1947). That vitamin A itself might be involved was indicated by Bearse et al. (1953). Subsequently, Scott et al. (1957) reported more blood-spot eggs from hens fed 800 I.U. of vitamin A per pound of diet than with hens fed higher levels. Bearse et al. (1960) reported the results of feeding experiments conducted with White Leghorn chickens. They found that: (1) blood-spot incidence was increased at vitamin A levels below 1,100 U.S.P. units per pound of feed; (2) vitamin A levels in the range of 1,200 to 1,600 units per pound resulted in minimum blood-spot incidence and that no further reduction occurred with levels up to 1,500 units; and (3) birds depleted of vitamin A reserves decreased in incidence of blood spots more rapidly when fed diets containing 3,600 and 10,000 units per pound than when fed diets containing 2,300 units per pound. Pope et al. (1961) conducted experiments in floor pens and in laying batteries with White Leghorns to determine the effects of certain minerals, vitamins and unidentified factor sources on blood-spot incidence. Sodium chloride, potassium chloride, sodium carbonate, their combination, vitamins A, E and K and corn fermentation products had no significant effect upon blood-spot incidence. Birds fed diets containing no

added vitamin A or very low levels produced a greater incidence of blood spots than did birds fed much higher levels. This defect was not associated with the individual hen's ability to mobilize vitamin A as determined by liver analysis. The authors further concluded that the extent to which blood spots occur is influenced by genetic differences, nutrition and bird age, as well as season, management and stress. March and Biely (1964) fed diets containing excessive amounts of vitamin A and vitamin K to laying hens. In both experiments, egg production was significantly reduced and there was no evidence that the blood-spot production was affected. This is in accord with the observations of Bearse et al. (1960) and Hill et al. (1961) who found that increasing the vitamin A level in the diet beyond the requirement for maximum egg production did not lower the blood spot incidence.

Since it has been shown that intrafollicular hemorrhages are responsible for blood spots, the theory has been postulated that extended blood-clotting time, as caused by dicumarol-like products, might cause an increase in such hemorrhages, thus resulting in a higher incidence of blood spot eggs. Waldroup and Harms (1962) added dicumarol, a vitamin K antagonist, to a layer diet and failed to significantly increase blood spotting. These

workers also concluded that blood prothrombin time was not related to the incidence of blood spots. Siddiqui and Fry (1963) studied the effect of warfarin in the diet of laying hens and reported that, while the blood prothrombin time was increased, the incidence of blood spots did not increase. The general conclusion, therefore, was that the incidence of blood spots and blood prothrombin times were independent. Lowered blood prothrombin time of layers, as a result of adding menadione sodium bisulfite complex (MSBC) to the diet, has been associated with an increased incidence of blood spots in eggs (Day and Woody, 1964). In contrast, the addition of anticoagulants to layer diets has reportedly prolonged blood clotting times without affecting the incidence of blood spots (Waldroup and Harms, 1962; Siddiqui and Fry, 1963). These authors theorized that eggs from hens treated with anticoagulants probably have blood in them but since clotting is slow, the blood is diffused throughout the albumen and not visible as a clot. Day et al. (1964) found that the incidence of blood spots in eggs was increased by sulfaquinoxaline (SQ) and decreased by dicumarol supplementation. Blood prothrombin time was significantly increased by dicumarol supplementation and tended to be increased by SQ. It is apparent from these data that the correlation, if any, between blood prothrombin times and the incidence of blood spots is dependent upon the causative agent.

Berruti and Didrick (1961) and Day and Woody (1964) reported that supplemental vitamin K decreased the incidence of blood spots in eggs. Sauter et al. (1963) indicated that supplemental alfalfa meal, a rich source of vitamin K, increases the blood spotting of eggs. Day and Woody (1964) reported an increase in the blood-spot incidence as a result of supplementing cage layer diets with MSBC or alfalfa meal. Blood prothrombin time was decreased with supplemental MSBC or alfalfa meal, suggesting a possible inverse relationship between prothrombin time of layers and blood-spot incidence. Fry et al. (1968) studied the relationship of vitamin K and vitamin A to blood-spot incidence and prothrombin times. They reported no definite relationship between prothrombin times and blood-spot incidence. Bearse et al. (1966) studied the effect of adding various levels of dehydrated and sun-cured alfalfa and dehydrated grass on blood-spot incidence. Different basal diets were formulated and fed, including one without any of the usual sources of vitamin K and containing sulfaquinoxaline. Regardless of amount or kind added, they failed to significantly ($P < 0.05$) increase the incidence or size of blood spots in the eggs produced. These results from birds maintained on litter-floored pens are in contrast to findings of Sauter et al. (1964, 1965) and of Day and Woody (1964) who observed the opposite effect

with birds maintained in wire-floored cages. Both these groups suggested that the rations fed resulted in marginal or deficient levels of vitamin K. Similarity in prothrombin times between basal and alfalfa supplemented diets indicates that the birds on the basal ration were receiving sufficient dietary vitamin K. Cage-maintained birds did not have supplementary vitamin K available from the litter. Sauter et al. (1964) conducted an experiment comparing 3 levels of dehydrated alfalfa meal and 3 levels of vitamin K supplied as MSBC in a corn-soybean meal basal diet. Blood-spot incidence increased for all dietary treatments. Approximately half of the hens in each dietary treatment produced no blood spots during the 10-week basal feeding period. In contrast, about 90% of all hens fed either alfalfa or vitamin K produced eggs with blood spots. Petersen et al. (1966) found that hens fed a vitamin K deficient diet produced significantly fewer blood spots than similar hens maintained on a corn-soy-alfalfa meal diet. The deficient diet, supplemented with either 3% alfalfa meal or vitamin K (748 µg per kilogram as MSBC) resulted in a 10% increase in blood spots. Vitamin K addition to the basal diet also resulted in a further increase of blood spots exceeding 15% of all eggs produced.

Recent work by Bearse (1962) has shown blood-spot incidence to increase as protein level of the feed is

increased from 12 to 18%. Pepper et al. (1967), while conducting experiments for the purpose of determining protein-calcium interaction in hens, observed that there was a significant increase in large blood spots as the protein level of high calcium diets was increased. The level of protein did not significantly affect the increase in large blood spots on the low calcium diets.

Since all the literature reviewed implicates the ovarian capillaries as the origin of blood found in blood spots, factors affecting the capillaries and connective tissue of birds have become of great interest. Shirley (1965) gives a detailed description of the formation of a blood spot during ovulation. After a laparotomy was performed on an anaesthesized hen, the rupturing of a capillary at the stigma was observed. The hemorrhage was judged to be beneath the surface layer of follicular tissue and occurred just prior to ovulation. In the study of any hemorrhagic disorder, the application of capillary resistance measurements is necessary in determining the nature of the bleeding. It would appear logical that the relative strength of capillaries would influence the occurrence of hemorrhages during ovulation. The cause of preovulatory, intrafollicular hemorrhage, resulting in blood spots, has not been determined; however, it is thought to be related to capillary fragility by many authors. Some reports have shown that hesperidin

and closely related compounds have a therapeutic effect in cases of capillary fragility of mammals (Scarborough and Edin, 1938; Sokoloff et al., 1957). Hudspeth et al. (1966), after feeding varying amounts of ascorbic acid and hesperidin to male chickens, found that, in most cases, both compounds tended to significantly decrease the strength of capillaries in the wing web as measured by a vacuum technique. Carver and Henderson (1948), contrary to suggestions by Nalbandov and Card (1947), found that the addition of rutin and vitamin C to the ration did not reduce the incidence of blood and meat spots. Vitamin P (bioflavonoids) in the form of lemon juice was ineffective (Nalbandov and Card, 1944) and rutin and ascorbic acid fed to chickens, likewise, failed to lower incidence of blood spots (Carver and Henderson, 1943).

One of the most dramatic changes in blood-spot incidence was reported by Bigland et al. (1964, 1965) who found that daily subcutaneous injections of water-soluble derivatives of flavone glucosides, chalcones, significantly reduced the incidence of blood spots. The derivatives of pyrrole-2-aldehyde chalcone reduced by two-thirds the incidence of a high blood-spot producing line. The same dose given orally had no significant effect. The chalcones used in these studies are believed to be the first compounds known that consistently reduce blood-spot incidence. The fact that chalcones have no effect on whole blood clotting time,

prothrombin time, hematocrit values, and erythrocyte count confirms the observations of Nalbandov and Card (1947) and Siddiqui and Fry (1963) that blood spots are not caused by a hemostatic defect.

High blood pressure could be a contributory stress in birds genetically susceptable to the high incidence of blood spots or in birds subjected to conditions which may reduce capillary resistance. Weiss (1958) examined the eggs from White Leghorn pullets whose systolic blood pressure differed widely and significantly in order to compare the incidence of blood spot formation. No statistically significant differences were found, suggesting that the normal range of pressure in the White Leghorn does not materially influence the occurrence of blood spots in eggs. While arterial and capillary pressure are generally correlated, they are not necessarily so, and hemodynamic controls localized in the ovary and oviduct could operate to maintain a uniform capillary pressure. Perhaps the complex venous drainage and spiral arteries of the follicle play such a role. Ward and Schaible (1963) report that the feeding of reserpine, an antihypertensive agent, had no influence on blood-spot incidence. Fry et al. (1968) reported the correlation coefficients of blood pressure with the percentage of blood spot eggs produced (+ .156), blood spot scores (+ .059), and average score of eggs containing blood (+ .096) from

223 birds. The authors concluded, from the positive correlation coefficients reported, that blood pressure is a factor in blood-spot incidence. However, since these correlation coefficients are quite small, the effect would appear to be minimal.

Chin and Brant (1953) reported that the addition of aureomycin to a laying diet showed some tendency to decrease the incidence of blood spots with added levels of antibiotic. These findings are essentially in agreement with those reported by Berg et al. (1952) using terramycin. Andrews et al. (1966) found that 14 and 16% protein diets supplemented with 0.02% arasanilic acid increased significantly the incidence of blood spots. To a lesser degree, this was also true of birds in floor pens at all protein levels used. This may be attributed to the increased requirement for vitamin K of caged layers fed arsanilic acid. The authors also reported that protein level was not associated with blood-spot incidence.

Any agent causing a hemorrhagic condition would be of interest as to their effect on the incidence of blood spots. Waibel and Pomeroy (1953) reported that β-aminoproprionitrile (BAPN) produced "dissecting aneurysm of the posterior aorta and, consequently, death due to internal hemorrhage" in growing turkeys. Barnett et al. (1958) reported that vitamin-K supplementation did not consistently

reduce BAPN-induced hemorrhaging in chickens, thus indicating that the BAPN effect is not mediated by causing a vitamin K deficiency. Ward and Schaible (1963) found that BAPN did not affect the number or size of blood spots produced. They concluded that the effect of BAPN on the small veins and capillaries of the ovary differs from its effect on the larger vessels, or it is not of sufficient magnitude to cause hemorrhaging.

CHAPTER II

RELATION OF COPPER DEFICIENCY TO BAPN TOXICITY

Introduction

Avian follicles are probably the fastest growing structures found in higher vertebrates. It is not surprising then, that, to accomplish the task of transportation and deposition of yolk material into the rapidly growing ova, a very complex circulatory system has been developed in this follicular area. Nalbandov and James (1949) classified the venous system of the follicles into 3 layers: (1) a capillary network in the theca of the follicle that drains by venules into (2) a complex network peripheral to the first layer that drains into (3) a third venous layer consisting mainly of a few large veins that drain into the follicular stalk. In contrast, the arterial supply is poorly developed, the system apparently depending on getting the blood into the venous network. When the largest follicle ovulates, spiral arteries providing the main blood supply in the follicle wall constrict when the ruptured follicle collapses; thus, the blood flow to the now empty follicular sac is greatly reduced and little, if any, bleeding occurs at ovulation. This is true even in the area of the torn

stigma through which the ovum is ovulated. The stigma itself is less vascular than the adjacent follicle wall but is by no means devoid of blood vessels (Nalbandov, 1964).

Bradley and Grahame (1950) described the circulatory system of the follicle as being a thick layer of dense connective tissue containing an abundance of capillaries lying beneath the germinal epithelium.

The functional importance of the connective tissue associated with the blood vessels of the follicle wall in facilitating the constriction of the spiral arteries at ovulation and the prevention of ruptures during growth is not known. The connective tissue of birds, especially that associated with the circulatory system, has been the subject of great interest recently. Both the presence of BAPN in the diet and the deficiency of copper in the diet have been studied in relation to their effect on the connective tissue of birds.

In 1961, Hill and Matrone, investigating the effect of a copper deficiency in chicks, reported that a great number of the copper-deficient chicks died even though the anemia was not severe. O'Dell et al. (1961) presented histological evidence which indicated there was a derangement in elastic tissue in the aortas of chicks fed a copper-deficient diet. The mortality found in these studies was

caused by a rupture of the major blood vessels. Subsequently, Carlton and Henderson (1963) and Simpson and Harms (1964) reported that the elastic membrane degenerated and aortic rupture occurred when the copper-deficient diet was fed. All of these findings clearly indicate that copper deficiency affected metabolism in a manner which was reflected in elastic tissue integrity.

Starcher et al. (1964) stated that the elastin content of the aorta of newly hatched chicks is approximately 5% of the wet weight of the aorta. When chicks were fed a diet containing 25 ppm copper, the elastin content increased to 12% by the seventeenth day. When the diet contained less than 2 ppm copper, the elastin content of the aorta increased more slowly and never equalled that of the control chicks. Amino acid analysis of elastin from copper-deficient and control chicks revealed that the lysine concentration of the copper-deficient elastin was 3 times that of control elastin. Hill et al. (1967) found similar results when following the content of aortic elastin from the day of hatch to four weeks of age in chicks fed a copper-deficient and a control diet. These workers reported that the results of copper deficiency could be reversed. After 16 days of copper supplementation to the diet of chicks that had been fed the deficient feed for 27 days, the elastin content of the aortas of these copper-deficient chicks had returned to normal.

Hill et al. (1967) reported that the lysine content of the elastin from copper-deficient chicks was much higher than that from the controls.

In 1963, an English group under the direction of Dr. S. M. Partridge isolated and proposed a structure of the cross-linkage groups in elastin (Thomas et al., 1963). This substance is called desmosine and its isomer isodesmosine; they are tetracarboxylic tetraamino acids. Partridge et al. (1964) obtained isotope data suggesting that desmosine could arise from the condensation of 4 lysine residues preexisting in straight-chain elastic precursors. In order for this reaction to take place, the authors proposed that the epsilon-amino group of the lysine residues would have to be removed and carbon oxidized, possibly to an aldehyde.

The role of copper in the conversion of lysine to desmosine is considered to affect the oxidative deamination of the epsilon amino acid group of the lysine residue. This type of reaction is catalyzed by a group of enzymes, amine oxidases, which contain copper.

Like all elastomers, natural or synthetic, elastin is essentially a cross-linked gel, and analysis of the stress-strain curves and other physical properties of moist elastin shows it to be composed of random coils which are kinetically free throughout the greater part of their length but are cross-linked by firm chemical bonds.

Hill et al. (1967) reported that chicks hatch without detectable amine oxidase activity in the aorta or liver. When the chicks were fed a diet containing copper, the activity appeared in both tissues by the third day at levels which were essentially maintained throughout the 26 days of the experimental period. When the diet was deficient in copper, the activity in the aorta remained undetectable throughout the period while, in the liver, activity remained substantially below that of the controls. These data are in accord with the hypothesis that the biochemical lesion responsible for the decreased content of aortic elastin in copper deficiency is a reduction in amine oxidase activity. This reduction in enzymatic activity ultimately results in fewer cross-linkages in the elastin which, in turn, results in less elasticity of the aorta and a general decrease in the strength of the elastin fiber bundles.

Rucker et al. (1968) found in studies with 10- and 20-day old chicks that low dietary copper (4 ppm) increased soluble bone collagen when compared with chicks fed adequate copper (25 ppm). The collagen content was determined by hydroxyproline concentrations in the soluble and insoluble protein fractions from femurs. Using benzylamine as substrate, these authors determined the level of amine oxidase activity of femurs from 10-day old chicks was 36% greater in controls

than deficient chicks. No differences were observed for the calcium, phosphorus, magnesium and copper content of bone ash from birds fed low copper diets.

It has been known for many years that the consumption of seeds from certain leguminous plants of the genus *Lathyrus* is associated with a disease that produces physical disability in humans. Following the isolation of the toxic substance from *Lathyrus odoratus* and its identification as β-aminopropionitrile (BAPN) (Schilling and Strong, 1955), this compound has been widely used to produce experimental lathyrism in various animals.

Barnett et al. (1957) first described the toxic effect upon turkey poults by feeding BAPN-HCl. The compound caused paralysis, degeneration of anterior motor neurons and growth depression when fed at levels of 0.12-0.25%, and pericardial and pulmonary hemorrhage, ruptured aortas, leg and toe deformities and growth depression at lower levels. Barnett and Morgan (1958) obtained similar results when chicks and chick embryos were used. They described in detail the anatomical lesions of the skeleton and vascular system induced by the BAPN. Roy and Bird (1959) found similar results when feeding 0.036% BAPN-fumarate to chicks. They reported a decrease in growth, a high incidence of leg deformities and some deaths due to ruptured aortas.

There are many reports in the literature that, in lathyrism, the amount of elastin in the large blood vessels is reduced just as it is in copper deficiency, and that dietary BAPN produces symptoms which are very similar to copper deficiency. Partridge (1966) reported that, in lathyrism, the amount of elastin in the large blood vessels is reduced and that feeding with BAPN produces symptoms which are very similar. Naber et al. (1967) studied the nature of BAPN-induced lathyrism and showed that the lathyrogen induced alterations in the molecular aggregation of collagen which increased the fragility and solubility of connective tissue. Page and Benditt (1967) agree that the lathyrogen appears to exert its effect on connective tissue by inhibiting the production of new covalent cross-links in collagen. They assayed, in the presence of BAPN, pig plasma amine oxidase, an enzyme thought to closely resemble the oxidase functional in collagen and elastin cross-linking. The BAPN was shown to inhibit enzyme activity competitively and reversibly forming a complex with the active site of the enzyme. It presently seems clear that BAPN prevents the formation of normal cross-links between the polypeptide chains that become a part of the collagen fibril. Consequently, connective tissue formed during BAPN intoxication in young animals is fragile and its collagen fibrils do not possess the normal tensile strength.

The hypothesis was formed, after a literature review concerned with blood-spot incidence in eggs, that this could be a manifestation of abnormal or deficient connective tissue in the follicle of the hen. An abnormality in the follicular connective tissue due to a deficiency of amine oxidase, for example, could account for the occurrence of ruptures in the vascular system of the follicle during rapid growth. A second possibility could be deficient connective tissue associated with the spiral arteries which would not sufficiently decrease the flow of blood at the time of ovulation. In both cases, the result could be the inherited characteristics of a high incidence of blood spots in an experimental strain of bird.

As a dietary means of testing this hypothesis, an experiment was devised using the effects of copper deficiency and BAPN toxicity to produce stress conditions upon the vascular connective tissue of an experimental high blood-spot incident strain. In theory, if the connective tissue of the blood-spot strain of bird was deficient in amount or integrity of connective tissue, these birds would be more susceptible to these stress conditions than would a normal strain of bird.

General Procedures

The experimental birds used in the following studies were a strain of Single Comb White Leghorn hens which produced a high percentage of blood-spot eggs. This strain was originally selected by the Western Washington Experiment Station (Puyallup) and has been maintained at the Florida Station since 1965. These birds will be referred to in the text as the blood-spot strain. Control birds were selected from a commercial strain of Single Comb White Leghorn.

In examining eggs for the presence of blood spots, each egg was broken out on a glass-topped stand. The yolk was separated from the albumen and its entire surface carefully examined under a magnifying lamp. The albumen was then checked for the presence of blood. Based on the maximum dimensions of the largest single inclusion of blood, the eggs were scored on the following scale: (0) - no blood; (1) - 1/16 inch or less; (2) - 1/16 to 3/16 inch; (3) - 3/16 to 1/2 inch; (4) - greater than 1/2 inch; and (5) - presence of blood in the albumen. Some subjective adjustment was made by increasing the score when large numbers of blood spots were present. The adjusted score was, therefore, more indicative of the total amount of blood present in the egg. All meat spots were eliminated from the scoring system. All eggs to be examined were stored at 55°F for no longer than 4 days and no less than 1 day.

All birds used, both the blood-spot and the control strains, were raised in the same manner. When not being fed an experimental diet, they were maintained on the standard farm feeds (Table 1) which were formulated using a corn-soybean meal base. Upon reaching maturity, all birds were placed in individual cages constructed to facilitate egg collection and identification and allowing for the maintenance of production records for each individual hen. Both the experimental and the standard farm feeds and water were fed ad libitum.

The birds used in the studies were individually selected on the basis of high production and a normal healthy appearance. The birds from the blood-spot strain were further selected on the basis of a high incidence of large blood spots. After selection, birds were randomly placed into the various groups used in the experiments.

Experiment I

The first experiment was conducted to ascertain the feasibility of using a copper-deficient diet and BAPN toxicity in comparing vascular integrity. These dietary methods were used to make comparisons between birds of the normal and blood-spot strain. Additionally, the relationship of feeding a low-copper parental diet to chick susceptibility to BAPN toxicity was observed.

TABLE 1

Standard farm formulas

Ingredients	% of diet		
	Starter	Grower	Layer
Yellow Corn Meal	62.85	81.60	69.85
Soybean Meal (50%)	31.00	12.25	19.00
Alfalfa Meal (20%)	2.50	2.50	2.50
Ground Limestone	1.00	1.00	6.00
Defluorinated Phosphate	1.90	1.90	1.90
Iodized Salt	0.25	0.25	0.25
Microingredient Mix[1]	0.50	0.50	0.50
% Protein	21.6	14.0	16.3
Productive Energy	977	1043	962
% Calcium	1.10	1.07	2.86
% Total Phosphorus	.73	.66	.67

[1]Supplied per kilogram of diet: vitamin A, 6600 I.U.; vitamin D_3, 2200 I.C.U.; vitamin K, 2.2 mg.; riboflavin, 4.4 mg.; pantothenic acid, 13.2 mg.; niacin, 39.6 mg.; choline, 499.4 mg.; vitamin B_{12}, 22 mcg.; Santoquin, 0.0125%; manganese, 71.4 mg.; iron, 19.8 mg.; copper, 1.98 mg.; cobalt, 0.198 mg.; iodine, 1.1 mg.; and zinc, 99 mcg.

Procedure

The experimental procedure was divided into two phases in preference to superimposing the stress of copper deficiency and BAPN (β-aminoproprionitrile) toxicity upon the vascular system of birds simultaneously. In the first phase, the effect of feeding the low-copper diet (Table 2) upon the production, fertility and hatchability of the blood-spot and normal strain of hen in the first year of production was determined. In the second phase, day-old chicks hatched during the first phase of the experiment were placed on a standard starter diet modified by increasing the added fat to 9% and containing 0.06% BAPN-fumarate (Table 3). Barnett and Morgan (1959) reported that mortality due to massive internal hemorrhages increased when the fat level in the diet was increased even though the chicks fed the high-fat diet consumed less feed. The parameters used in determining the effect of BAPN toxicity in this phase of the study were toe and leg deformities, mortality, incidence of aortic ruptures and body weight. Following this two-phase experimental procedure, the predisposition of the chicks from hens fed the low-copper diet to the toxicity of BAPN could be determined. Three consecutive studies were conducted following this general experimental procedure.

In the first study, 5 groups of laying hens of 20 birds each were used. These groups consisted of blood-spot

TABLE 2

Composition of low-copper layer diet[1]

Ingredients	% of diet
Dried skim milk	50.0
Cerelose	31.0
Corn oil	5.0
Glycine	0.5
DL-methionine	0.3
L-arginine	1.0
Choline Chloride (50%)	0.1
Vitamin A (250,000 10/gm)	1.0g
Vitamin D_3 (3',000 1CU/gm)	3.5g
Vitamin E (20,000 10/lb)	20.0g
Santoquin (67%)	8.0g
NaH_2PO_4 (22.45%)P	4.0
$CaCO_3$ (40% Ca)	6.0
Microingredients[2]	0.5
Salt premix[3]	1.5

[1]By analysis, diet contained 2ppm copper.

[2]Furnishes per Kg diet (mg/ky): 0.03 vitamin B_{12}, 0.30 biotin, 1.0 menadione, 8.0 pyridoxine HCL, 4.0 folic acid, 16.0 riboflavin, 100 nicotinic acid, 20 calcium pantothenate, 24 thiamine.

[3]Furnishes per pound of premix (grams): 9.1 $MnSO_4$, 90.7 $MgSO_4$, 90.7 iodized salt, 18 $FeSO_4 \cdot 7H_2O$, 0.33 $ZnCl_2$, 0.013 $CoCl_2$, 137KCl.

TABLE 3

BAPN starter diet

Ingredients	% of diet
Yellow corn	50.4
Soybean meal (50% protein)	25.0
Alfalfa (20% protein)	3.0
Corn gluten	4.5
Animal fat	9.0
Fish meal	5.0
DL-methionine	22.7 g
Defluorinated phosphate	2.0
Ground limestone	0.2
Iodized salt	0.4
Vitamin premix[1]	0.5
β-aminopropionitrile	0.06

[1]Supplied per kilogram of diet: vitamin A, 6600 I.U.; vitamin D_3, 2200 I.C.U.; vitamin K, 2.2 mg.; riboflavin, 4.4 mg.; pantothenic acid, 13.2 mg.; niacin, 39.6 mg.; choline, 499.4 mg.; vitamin B_{12}, 22 mcg.; Santoquin, 0.0125%; manganese, 71.4 mg.; iron, 19.8 mg.; copper, 1.98 mg.; cobalt, 0.198 mg.; iodine, 1.1 mg.; and zinc, 99 mcg.

and normal hens—each inseminated using semen from normal males. A third group was composed of blood-spot hens inseminated from blood-spot males. These 3 groups were supplied the semisynthetic low-copper diet. The remaining 2 groups consisted of blood-spot hens and normal hens—each inseminated from their respective males. These last 2 groups were fed the regular farm laying feed and served as a standard in comparing the production, fertility and hatchability during the experimental period.

All inseminations were made using 0.05 ml of pooled semen. The first 2 inseminations were made at a 2-day interval and thereafter the birds were inseminated every 7 days during the experimental period. The first 3 groups were fed the low-copper diet for an 18-day period immediately following the second insemination. After this feeding period, they were placed back on the regular farm laying diet for a period of 12 days. Extensive precautions were taken during the mixing and feeding of the semisynthetic low-copper diet to prevent its adulteration with copper ions. All mixing apparatus and feed containers were carefully washed, rinsed with a chelating agent and given a final rinse with deionized, distilled water prior to use. Both the feed and water troughs were lined with polyethylene which had been washed and rinsed as described above. During the 30-day experimental period, eggs were collected daily,

marked for identification with the date and hen number and incubated every 3 days. Eggs were candled on the fifth and eighteenth day of incubation.

As the chicks hatched every 3 days, they were wing-banded and placed in wire-floored batteries. All chicks from the 5 groups were fed the modified starter diet containing BAPN for a 5-week period. Each day the groups were checked and the dead birds posted to determine if death could be attributed to aortic rupture. At the end of the 5-week growing period, the surviving birds were visually scored for severity of leg and toe disorder and weighed. The severity of disorders for each bird was graded using a scale from 1 to 3 for each leg. A score of 1 indicated the absence of any disorder and a score of 3 indicated the most severe cases. Scores for the legs were added; therefore, each bird received a score of from 2 to 6. The birds were weighed by groups and sex, the latter determined by secondary sex characteristics.

Results and Discussion

Production for the various groups are shown in Table 4 and are presented as percentages based on the number of hens during each 3-day period. The final number of birds used as a source of data for each group was dependent upon the health and performance of the bird during the entire

TABLE 4

Percent egg production of normal and blood-spot hens fed a copper-deficient diet

Cross M	Cross F	No. Birds	3-day periods: Treatment (18 days) Diet	1	2	3	4	5	6	Post-treatment (12 days) Diet	7	8	9	10
BS × BS[1]		19	Cu^{-}[3]	50.9	33.3	38.6	42.1	29.8	19.3	L	33.3	45.6	40.4	47.4
BS × BS		14	L[4]	64.3	57.1	50.0	42.9	50.0	50.0	L	38.1	45.2	59.5	42.9
N × N[2]		8	Cu^{-}	66.7	50.0	41.2	37.5	50.0	41.2	L	29.2	33.3	45.8	58.3
N × N		22	L	62.1	65.2	66.7	51.5	63.0	62.1	L	65.2	65.9	59.1	65.2
N × BS		16	Cu^{-}	50.0	43.8	43.8	31.3	25.0	25.0	L	22.9	18.8	39.6	54.2

[1]Blood-spot strain.

[2]Normal commercial strain.

[3]Semisynthetic low-copper diet.

[4]Standard farm layer diet.

30-day period. All data collected from a bird which went into a molt, died or failed to produce a single fertile egg after the first 2 inseminations were eliminated from the statistical analysis. The final number of birds used in each group as a source of data are listed in the table.

The production of the normal x normal and blood-spot x blood-spot crosses which remained on the farm laying feed was comparable and remained constant, indicating no definite trends. Any large differences in the production of these groups from one 3-day period to the following may be attributed to the stress of handling during insemination and normal variation expected when using relatively small numbers of birds in each group. The actual production percentages themselves are not as important as the trends which they indicate.

The production in all groups fed the low-copper diet decreased. This decrease in production is most evident in the normal x normal cross. The normal x blood-spot cross group displayed the lowest production rate during the second 3-day period after removal from the low-copper diet. In all groups fed the low-copper diet, production returned to the pretreatment level after feeding the commercial type diet for a 12-day period.

Using the number of eggs laid during the 3-day periods for individual hens in the groups as an observation,

an analysis of variance was conducted (Snedecor, 1961). The production rates for the groups, periods and the group x period interaction were all significantly different at the 0.1% level. The interaction of group x period indicated that as the copper reserves in the hen's body were depleted on the low-copper diet, production decreased significantly.

In Table 5, the means and the standard errors for each group are given. It can be seen that the production rates for both the normal x normal and blood-spot x blood-spot crosses were significantly lowered when fed the low-copper diet as compared to the same cross fed the farm feed. No difference was found in the effect of the low-copper diet upon the normal x blood-spot and blood-spot x blood-spot crosses.

The fertility and the hatchability of the fertile eggs in each of the 5 groups during the ten 3-day periods are listed in Tables 6 and 7, respectively. When the incidence of infertile eggs was analyzed using chi-square, it was found that while the occurrence in the blood-spot group was significantly greater ($P < 0.001$) than in the normal x normal group on the farm feed, only the normal birds were affected by the dietary treatment. The increase in incidence of infertile eggs in the normal x normal group on the low-copper diet compared to the normal x normal control group was significant at the 0.5% level. In the

TABLE 5

Average number of eggs produced per hen during 30-day experimental period

	6-day groups				
Group	1	2	3	4	5
Cross	BS × BS[1]	BS × BS	N × N[2]	N × N	N × BS
Diet	Cu⁻[3]	L[4]	Cu⁻	L	Cu⁻
Mean[5]	11.11^{a}	17.17^{bc}	13.63^{b}	18.23^{c}	10.88^{a}
SEM[6]	0.92	1.33	1.56	1.22	1.02

[1]Blood-spot strain.

[2]Normal commercial strain.

[3]Semisynthetic low-copper diet.

[4]Standard farm layer diet.

[5]The average number of eggs produced per hen in each group; means with different superscript are significantly different.

[6]Standard error of the mean.

TABLE 6

Percent fertility in normal and blood-spot hens fed a copper-deficient diet

Cross M	Cross F	No. Birds		3-day periods: Treatment (18 days) 1	2	3	4	5	6		Post-treatment (12 days) 7	8	9	10
BS × BS[1]		19	Cu^{-}[3]	77.8	94.7	85.7	83.3	88.2	72.7	L	68.4	92.0	100	85.2
BS × BS		14	L[4]	96.2	87.0	76.2	77.8	85.0	70.0	L	75.0	84.2	64.0	72.2
N × N[2]		8	Cu^{-}	93.3	100	80.0	77.8	83.3	80.0	L	42.9	100	90.0	71.4
N × N		22	L	95.0	97.6	100	100	93.4	97.6	L	83.7	96.6	94.9	88.4
N × BS		16	Cu^{-}	91.3	100	95.2	100	90.9	91.7	L	90.9	100	84.2	80.8

[1]Blood-spot stain.

[2]Normal commercial strain.

[3]Semisynthetic low-copper diet.

[4]Standard farm layer diet.

TABLE 7

Percent hatchability of fertile eggs from normal and blood-spot hens fed a copper-deficient diet

Cross		No.	3-day periods										
			Treatment (18 days)						Post-treatment (12 days)				
M	F	Birds	Diet	1	2	3	4	5	6	Diet	7	8	9	10
BS × BS[1]		19	Cu⁻[3]	57.1	77.8	66.7	95.0	93.3	75.0	L	69.2	87.0	91.3	82.6
BS × BS		14	L[4]	80.0	70.0	87.5	92.9	76.5	71.4	L	58.3	68.8	93.8	69.2
N × N[2]		8	Cu⁻	84.6	100	100	100	100	100	L	33.3	87.5	100	80.0
N × N		22	L	97.4	92.7	95.5	100	94.4	92.5	L	83.8	92.9	94.4	94.7
N × BS		16	Cu⁻	95.2	89.5	84.2	80.0	80.0	45.5	L	70.0	38.9	75.0	81.0

[1]Blood-spot strain.

[2]Normal commercial strain.

[3]Semisynthetic low-copper diet.

[4]Standard farm layer diet.

normal x normal group during the first 3-day period after being fed the low-copper diet, the fertility decreased to 42.9% which represents approximately 50% of the initial fertility. The dietary treatment did not affect fertility of the blood-spot strain. The occurrence of infertile eggs in the normal x blood-spot group decreased to a level between that of the 2 parent strains and near that of the normal x normal group.

The trend for hatchability of fertile eggs was similar to that of fertility with the normal x normal cross treated with the low-copper diet showing a large decrease during the first 3-day period after being placed back on the control diet. The normal x blood-spot cross showed a steady decrease in hatchability with length of time fed the experimental diet, dropping from 95 to 45% in 18 days. Analysis of the data by chi-square showed significant differences between the hatchability of fertile eggs in the 5 groups at the 0.5% level. The normal birds had a significantly greater ($P < 0.05$) hatch than did the blood-spot birds. However, in each cross, the feeding of the low-copper diet did not affect hatchability. Hatchability of the normal x blood-spot cross was almost identical to that of the blood-spot x blood-spot cross fed the low-copper diet.

The results of the first phase of this study are not in agreement with those of Simpson et al. (1967) who

reported a decrease in hatchability to 0 in 10 days when hens were fed a similar low-copper diet. These authors further reported that feeding a low-copper diet had no significant effect on production when using a normal strain of bird.

The 5-week weight of the chicks placed on the starter diet containing 0.06% BAPN are presented as the average weight for each group during a 3-day period (Table 8). The analysis of variance showed that the period effect was significant at the 1% level and that the period x cross interaction was significant at the 5% level. These factors were the only two found to be significant. There was no detectable difference in body weight due to sex. It does not appear that the low-copper parental diet affected the chick weights to any great extent. The chicks from the blood-spot hens, while not significantly different in weight from those of normal hens, were heavier regardless of the parental diet. The lowest chick weights recorded were from the normal x normal cross and occurred when hens were placed back on the parental control diet. These chicks were from the same group and period which had the lowest fertility and hatchability in the first phase of this study.

The scores indicating the severity of leg and toe deformities of the chicks are presented in Table 9. No differences were detected in these scores as a result of

TABLE 8

Five-week weight of chickens fed a starter diet containing 0.06% BAPN from hens fed a low-copper diet

Cross	Diet		3-day periods: Parental treatment 1	2	3	4	5	6	Post parental treatment: Diet		7	8
BS × BS[1]		M[5]	334[8]	337	305	296	267	247		M	251	227
	Cu^-[3]	F[6]	315	303	273	290	284	295	L	F	170	213
		A[7]	325	320	289	293	276	271		A	211	220
BS × BS		M	318	271	266	326	279	309		M	268	245
	L[4]	F	301	281	270	303	311	289	L	F	150	214
		A	310	276	268	315	295	299		A	209	229
N × N[2]		M	282	287	269	282	283	294		M	188	225
	Cu^-	F	296	243	145	318	293	273	L	F	. .	242
		A	289	265	207	300	288	284		A	188	233
N × N		M	269	291	266	294	281	307		M	257	. .
	L	F	276	269	266	292	271	309	L	F	250	238
		A	272	280	266	293	276	308		A	253	238
N × BS		M	277	323	300	297	323	325		M	. .	208
	Cu^-	F	328	284	231	299	304	317	L	F	268	148
		A	302	303	265	298	313	321		A	268	178

[1]Blood-spot strain.

[2]Normal commercial strain.

[3]Low-copper diet.

[4]Standard farm layer diet.

[5]Males

[6]Females

[7]Averaged male and female group.

[8]Weight in grams.

TABLE 9

Severity of leg and toe deformities in chicks at 5 weeks of age

Cross M	Cross F	Parental diet	Average for 3-day periods: Treatment 1	2	3	4	5	6	Parental diet	Post treatment 1	2
BS × BS[1]		Cu⁻[3]	3.6[5]	4.5	2.5	3.4	2.3	3.3	L	2.6	4.0
BS × BS		L[4]	4.4	3.9	2.9	2.8	2.9	3.2	L	2.7	3.1
N × N[2]		Cu⁻	5.1	4.3	4.7	2.5	3.3	4.3	L	4.0	2.3
N × N		L	4.6	3.2	3.0	3.0	3.2	3.9	L	2.3	2.8
N × BS		Cu⁻	3.5	4.4	3.7	2.0	2.0	3.8	L	2.0	3.0

[1]Blood-spot strain.

[2]Normal commercial strain.

[3]Semisynthetic low-copper diet.

[4]Standard farm diet.

[5]Numerical score from 2 (normal) to 6 (most severe).

parental treatment or strain of chick. Over 80% of the birds had deformities which were attributed to the BAPN toxicity.

The incidence of death and deaths specifically caused by the aortic rupture during the 5-week period on the BAPN diet were relatively low and are shown in Table 10. The post mortem examination of the dead birds showed that only a moderate percent of the deaths could be attributed to a rupture of the aorta.

The incidence of death in the 5 groups as analyzed by chi-square were significantly different at the 5% level, indicating that the normal chicks were more susceptible to the effects of BAPN than were the blood-spot chicks regardless of dietary treatment. The incidence of death due to aortic rupture was significant at the 0.5% level and the normal x normal cross again appeared more susceptible than the blood-spot strain to the toxic effects of BAPN. No treatment differences between the 2 groups of each cross were detected indicating that the parental diet had little effect upon the susceptibility of the chicks.

Experiment II

The second study was conducted to clarify those results previously obtained. The unexpected loss in production of hens fed a copper-deficient diet had not previously been

TABLE 10

Mortality of chicks fed a starter diet containing 0.06% BAPN at 5 weeks of age

Cross			3-day periods								
			Parental treatment							Post treatment	
M	F	Diet	1	2	3	4	5	6	Diet	1	2
BS	BS	Cu^{-} [1]	16	15	12	19	14	6	L [1]	9	17
		[2]	0	2	2	1	2	1	[2]	0	4
		[3]	0	0	0	0	0	0	[3]	0	2
BS	BS	L [1]	21	14	14	14	14	10	L [1]	7	11
		[2]	1	0	1	1	1	2	[2]	1	1
		[3]	1	0	1	1	0	0	[3]	1	0
N	N	Cu^{-} [1]	19	14	8	7	12	8	L [1]	3	9
		[2]	3	1	3	1	2	0	[2]	1	2
		[3]	0	0	1	0	1	0	[3]	1	0
N	N	L [1]	38	40	43	36	. . [4]	38	L [1]	16	5
		[2]	3	8	7	5	. .	10	[2]	6	1
		[3]	1	2	2	1	. .	6	[3]	2	0
N	BS	Cu^{-} [1]	21	17	17	12	8	5	L [1]	6	11
		[2]	2	2	3	6	1	1	[2]	3	4
		[3]	1	0	1	1	0	0	[3]	3	3

[1] Initial number of chicks.

[2] Number of deaths.

[3] Number of deaths attributed to aortic rupture.

[4] No data collected.

reported in the literature; thus, it was necessary to repeat the first study in an attempt to substantiate those results.

Procedure

The same general procedure outlined in the first study was followed with only a few minor changes. The number of groups used was reduced to 4 with 20 birds each. Groups consisted of 2 of the normal and 2 of the blood-spot strain crossed with their respective males. One group of each cross was fed the low-copper diet during the first 15 days of the 30-day experimental period. The experimental diet was found by analysis to contain the same level of copper present in the diet of the first study, 2 ppm. The practical type diet was not analyzed. The remaining group of each strain served as the control and remained on the regular farm laying feed. To eliminate a possible source of copper ions, all birds were provided with deionized, distilled water ad libitum. During the second phase of the experiment, the chicks were raised in floor pens on peanut hull litter. This procedural change was made to eliminate the influence of the wire floor in the batteries upon the occurrence and severity of leg and toe disorders. The amount of BAPN added to the modified starter diet was increased from the previous level of 0.06 to 0.1% in an effort to increase the incidence of aortic ruptures. The

chicks were maintained on this diet for 21 days at which time the study was terminated. Body weights were obtained by groups according to treatment (cross and diet) for each 3-day period.

Results and Discussion

The production of the 4 groups during each of the ten 3-day periods are presented in Table 11. The analysis of variance for the number of eggs laid by individual hens during each 3-day period revealed that the effects of treatment, periods and the treatment x period interaction were all significant at the 1% level. Applying the error of the mean squared showed that the average production of the 4 groups during the 30 days were all significantly different ($P < 0.01$). The normal group of hens maintained on the control diet had the highest rate of production. Feeding the copper-deficient diet resulted in the greatest change in production to the normal group, decreasing from 81 to 9% after only 6 days. Production remained at this low level until the birds had been returned to the control diet for a period of 4 days. The control group of blood-spot birds produced at a significantly lower rate than the normal strain of controls. While feeding the low-copper diet significantly decreased production in both strains, the decrease observed in the blood-spot strain was not as great

TABLE 11

Percent egg production of normal and blood-spot hens fed a copper-deficient diet

Cross		No.	3-day periods										
			Treatment (15 days)						Post-treatment (15 days)				
M	F	Birds	Diet	1	2	3	4	5	Diet	6	7	8	9	10
BS × BS[1]		15	L[3]	82.2	80.0	73.3	75.0	75.0	L	64.4	73.3	53.3	64.4	53.3
BS × BS		15	Cu^{-}[4]	73.3	48.9	42.2	37.8	35.6	L	53.3	60.0	62.2	68.9	68.9
N × N[2]		18	Cu^{-}	81.5	33.3	9.3	3.7	5.6	L	7.4	29.6	55.6	83.3	79.6
N × N		19	L	79.0	82.5	63.2	94.7	86.0	L	79.0	89.5	77.2	77.2	77.2

[1]Blood-spot strain.

[2]Normal commercial strain.

[3]Standard farm layer diet.

[4]Semisynthetic low-copper diet.

as that observed in the normal strain. Production of the blood-spot group dropped to only 35.5% from the initial rate of 73.3%. Production of both treated groups returned to the initial rate after being placed back on the control diet for 9 days.

The fertility and hatchability of these 4 groups during each 3-day period are given in Tables 12 and 13, respectively. The chi-square analysis of the fertility data showed that only the dietary treatment effects were significant ($P < 0.01$). While it is not evident from the percentages given in Table 12, the only significant difference ($P < 0.01$) was the decrease in fertility of the blood-spot group when fed the low-copper diet. The decrease in fertility of the normal strain of birds during the last 3 days on the experimental diet was not shown to be significant. The production of this group during the seventh to the fifteenth days on the low-copper diet was extremely low; this greatly influenced the weight given to the fertility and hatchability results. The percent fertility and hatchability during the last 3 days on the low-copper diet were based on a total of 3 eggs laid by the 18 birds in the normal group. No significant differences were found in the hatchability of the 4 groups.

The analysis of variance of the chick weights at 21 days of age showed them to be significantly different

TABLE 12

Percent fertility of normal and blood-spot hens fed a copper-deficient diet

Cross		No.	3-day periods										
			Treatment (15 days)						Post-treatment (15 days)				
M	F	Birds	Diet	1	2	3	4	5	Diet	6	7	8	9	10
BS × BS[1]		15	L[3]	88.2	97.1	97.0	100	97.1	L	93.1	97.0	100	93.1	100
BS × BS		15	Cu^{-}[4]	93.9	91.0	84.2	82.4	81.3	L	87.5	100	92.9	93.5	93.5
N × N[2]		18	Cu^{-}	90.9	100	100	100	86.7	L	100	93.8	92.9	97.7	97.7
N × N		19	L	95.6	93.6	91.7	96.3	95.9	L	97.8	95.8	90.9	93.0	95.4

[1]Blood-spot strain.

[2]Normal strain.

[3]Standard farm layer diet.

[4]Semisynthetic low-copper diet.

TABLE 13

Percent hatchability of fertile eggs from normal and blood-spot hens fed a copper-deficient diet

Cross	No. Birds		3-day periods									
			Treatment (15 days)					Post-treatment (15 days)				
		Diet	1	2	3	4	5	Diet	6	7	8	9	10
BS × BS[1]	15	L[3]	100	94.1	90.6	97.1	93.9	L	92.6	90.6	91.7	74.1	95.8
BS × BS	15	Cu^{-}[4]	100	90.0	87.5	100	84.6	L	90.5	88.9	96.2	96.6	93.1
N × N[2]	18	Cu^{-}	100	100	100	100	100	L	100	100	88.5	97.6	92.9
N × N	19	L	97.7	95.5	93.9	94.2	95.7	L	93.2	97.8	97.5	95.0	95.1

[1]Blood-spot strain.

[2]Normal commercial strain.

[3]Standard farm layer diet.

[4]Semisynthetic low-copper diet.

at the 1% level. These weights are given in Table 14. The birds in both normal groups were significantly ($P < 0.01$) heavier than the blood-spot birds in the remaining groups. In both groups fed the low-copper diet, growth was significantly depressed ($P < 0.01$) when each strain was compared to its respective control group. No differences were detected in the effects of the parental diet on the susceptability of the chick to the toxicity of the BAPN in the first study. This difference in the results of the 2 studies could possibly be affected by the increased level of BAPN used in the second study. The higher concentration of BAPN surpassed the threshold level of both strains and, thus, failed to indicate the differences previously observed. The possibility that the differences in body weight can not be detected until after the third week of age may also be another consideration. The chicks from all 4 groups hatched the last 3 days were placed on regular farm starter diets without the addition of BAPN and, thus, served as positive controls. Chicks of the normal strain weighed more than those of the blood-spot strain. All 4 groups of the positive control chicks were 20 g or approximately 20% heavier at 21 days than the comparable groups fed the BAPN-treated diet.

No differences were found in the occurrence or severity of leg and toe disorders between birds of the

TABLE 14

Three-week weight of chickens fed a starter diet containing 0.1% BAPN from hens fed a low-copper diet

		3-day periods									
		Parental treatment					Post-parental treatment				Control[6]
Cross	Diet	1	2	3	4	5	Diet	6	7	8	9	birds
BS × BS[1]	L[3]	21[5]	21	23	20	18	L	17	19	17	21	20
		109[7]	115	136	110	97		102	98	108	111	128
BS × BS	Cu⁻[4]	21	20	13	13	7	L	16	16	15	20	20
		112	109	122	109	90		98	105	114	109	129
N × N[2]	Cu⁻	22	12	5	2	2	L	3	8	13	19	23
		111	114	139	107	108		112	93	109	114	137
N × N	L	17	14	21	21	23	L	19	11	18	19	24
		114	118	140	112	106		106	106	122	122	138

[1]Blood-spot strain.

[2]Normal commercial strain.

[3]Standard farm layer diet.

[4]Low-copper diet.

[5]Number of chicks.

[6]Chicks fed the starter diet without BAPN.

[7]Weight in grams.

4 groups. The amount of variation inherent in the subjective scoring method was probably so large that the sensitivity may have not been great enough to detect differences between groups if they did exist. The 2-week difference in age of the chicks at the time they were weighed and scored complicated comparisons between those in the first study raised in batteries and those in the second study raised on the floor. However, there appeared to be no increase in these disorders as a result of increasing the level of BAPN in the diet.

Since the chicks of the 4 groups in each 3-day period were intermingled, no data were obtained on feed consumption. The incidence of leg and toe disorders reduced the ability of the birds to reach food and water; however, the actual reduction in feed intake could not be determined. This effect probably accounted for the greater variation in the weight of the birds in the first study at 5 weeks of age as compared with the variation in weight of the second study at 3 weeks of age.

Mortality and the incidence of aortic rupture in the 4 groups during the 21-day growth period were analyzed by chi-square. No significant differences between the groups were detected for either mortality or aortic rupture. These percentages are given in Table 15 and compare favorably with those reported by Barnett and Morgan (1959). The

TABLE 15

Mortality of chicks fed a starter diet containing 0.1% BAPN at 3 weeks of age

Cross				3-day periods								
				Parental treatment					Post-parental treatment			
M	F	Diet		1	2	3	4	5	Diet	6	7	8	9
BS × BS		L	[1]	24	24	24	24	24	L	22	24	22	22
			[2]	3	3	1	4	6		5	5	5	1
			[3]	0	0	1	4	0		1	3	2	0
BS × BS		Cu⁻	[1]	24	22	14	14	11	L	19	23	24	24
			[2]	3	2	1	1	4		3	7	5	4
			[3]	0	0	0	0	0		1	0	1	0
N × N		L	[1]	24	18	5	2	2	L	4	15	22	24
			[2]	3	5	0	0	0		1	7	7	5
			[3]	0	0	0	0	0		1	0	1	2
N × N		Cu⁻	[1]	24	24	23	24	24	L	24	24	24	23
			[2]	6	11	2	3	1		5	13	5	4
			[3]	1	2	2	2	0		0	0	1	0

[1] Initial number of chicks.

[2] Number of deaths.

[3] Number of deaths attributed to aortic rupture.

increased level of BAPN in the diet did not noticeably increase either the mortality or the incidence of aortic rupture when compared with the first study. The incidence of death and death resulting from aortic rupture are plotted against the age of the bird in Figure 1. Aortic ruptures were not detected until the sixteenth day and the incidence increased until the experiment terminated. The direct relationship, shown in the graph, between the incidence of death and death attributed to aortic rupture indicates that a much larger number of deaths were probably the result of aortic or other vascular disorders than could be detected by gross post-mortem examination.

It can be concluded from these results, as it was in the first study, that no vascular weakness or abnormalities were detected in the blood-spot strain as a result of the stress placed upon them by feeding a copper-deficient diet or from the BAPN toxicity. The results of this second study confirm those of the previous one--that the blood-spot strain of birds is not as susceptible as the normal strain to the stress of a copper-deficient diet. The response of the 2 strains of chicks to the stress of the BAPN was not significantly different.

The strain of bird involved in nutritional studies using semisynthetic or purified diets has a great effect upon the results obtained (Wesheim, 1968). Variance in the

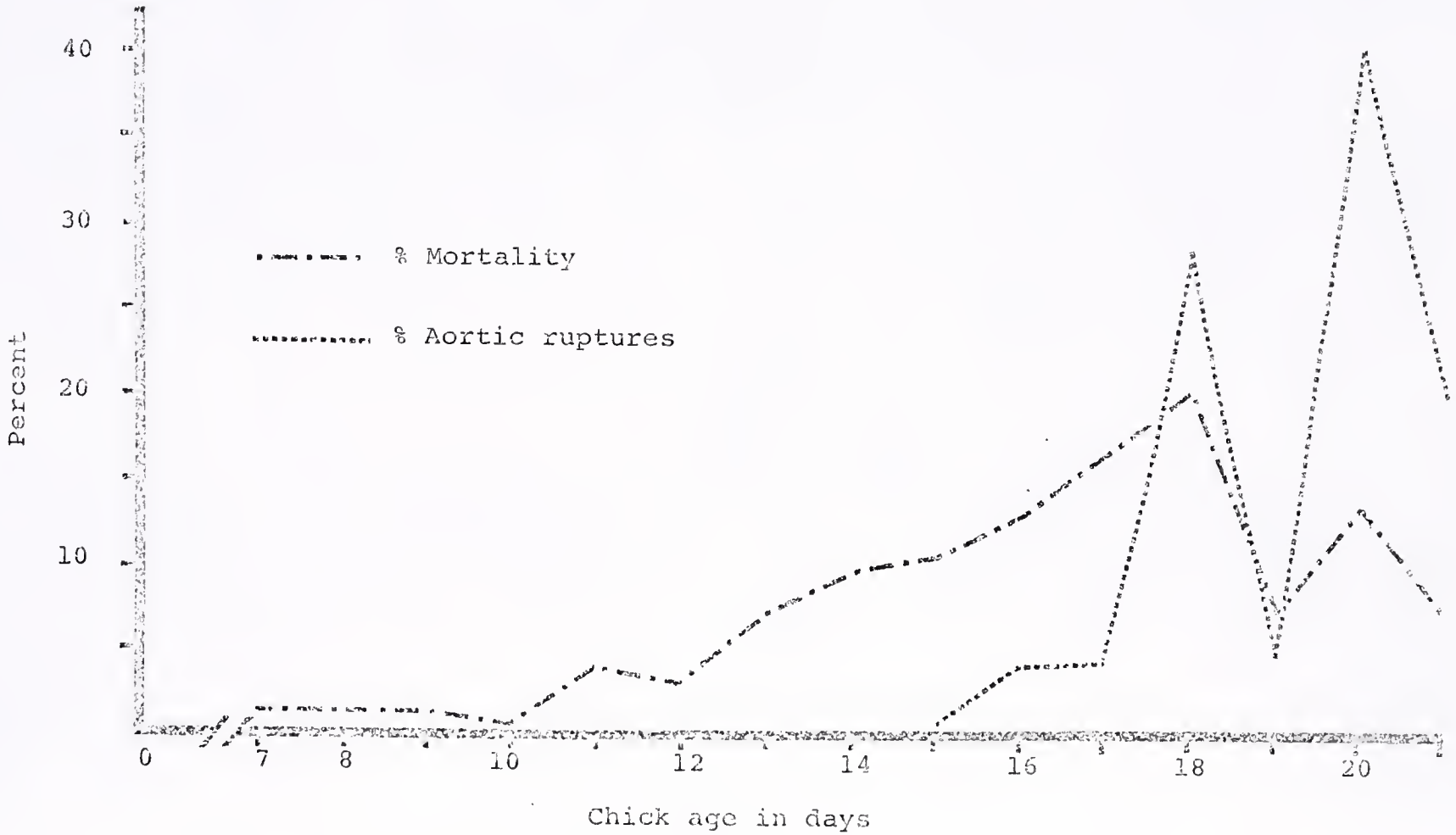

Fig. 1 Aortic rupture and mortality of chicks from both strains fed a diet containing 0.1% BAPN.

nutritional requirements between strains of birds is well documented (Kondra and Hodgson, 1961, and Balloun and Speers, 1969).

Without further evidence, the differences in nutritional requirements between the normal and blood-spot strain cannot be directly or indirectly related to the factors which result in the high incidence of blood-spot eggs produced.

Experiment III

A third study was conducted using a copper-supplemented, semisynthetic diet as the control in ascertaining the effect of a low-copper diet upon laying hens. In previous studies, the commercial type diet served as the control. To obtain a better indication of the effects of this low-copper diet, analyses were conducted to determine liver-copper levels. Furthermore, the aortas of the chicks hatched during the first phase were examined at 1 day of age for the presence of abnormalities in the morphology of the connective tissue.

Procedure

The procedure followed that used in the previous study. A total of 28 birds were used, 14 from both the high blood-spot incident and normal strains. Half the birds

of each strain were fed the low-copper diet (basal) for a period of 18 days. The remaining 7 hens from each strain were fed the basal diet supplemented with 16 ppm of copper as copper sulfate. Dietary analyses showed that the basal contained 3.0 ppm copper and the supplemented 18.0 ppm copper. Production, fertility and hatchability were determined during the 18-day period. At the end of the experimental time, the hens were exanguinated and the intact livers excised. Each liver was placed in a polyethylene bag and frozen in a blast freezer at -18°C where they were subsequently stored. The livers were prepared for analysis by partially thawing before dicing with surgical scissors. Each diced liver was then equally divided into 2 crucibles. Samples were dried overnight at 110°C, cooled in a dessicator and the dry weights obtained. Using concentrated nitric acid, the solid material was digested by boiling on a hot plate. The crucibles were heated until contents were almost dry and then placed in a muffle furnace for ashing. After reaching 200°C, the temperature was raised 100° hourly until the desired 600°C was reached. This temperature was maintained for 12 hours to complete ashing. The ash was then dissolved in boiling 10% hydrochloric acid, filtered and diluted to a known concentration. The iron and copper levels were determined using an atomic absorption spectrophotometer according to the method recommended by the manufacturer (Anonymous, 1964).

It has been shown that hens fed a low-copper diet produce chicks with abnormalities of the vascular system (Simpson et al.,1967). However, it is not known if these abnormalities are corrected by feeding a normal starter diet during the first several weeks of growth. In Experiment III, half the chicks hatched from the 4 groups of hens during each 3-day period were killed at 1 day of age. The hearts and aortas were excised and placed in 10% neutralized formalin until prepared for examination using a Philips EM 200 electron microscope following the procedure of Simpson et al. (1967). The remaining chicks were maintained in batteries for 21 days and fed the regular practical type diet. When they reached 21 days of age, they were killed and the hearts and aortas removed for comparison with those removed from birds of the same group and period at 1 day of age.

Results and Discussion

The rates of production for the 4 groups during each 3-day period are given as percentages in Table 16. The analysis of variance showed that the periods were significantly different ($P < 0.01$) and that the period x treatment interaction was significant at the 5% level. While these results show a definite dietary effect, the number of birds used in each group was too low to show

TABLE 16

Percent egg production of normal and blood-spot hens fed a copper-deficient diet

M	F	Diet	No. Birds	Averages for 3-day periods					
				1	2	3	4	5	6
BS	BS[1]	Cu^{+}[3]	5	40.0	46.7	40.0	33.3	33.3	45.0
N	N[2]	Cu^{+}	6	60.1	66.7	55.6	44.4	55.6	66.6
BS	BS	Cu^{-}[4]	5	46.7	40.0	46.7	53.3	53.3	50.0
N	N	Cu^{-}	7	71.4	71.4	66.7	42.9	28.6	21.4

[1]Blood-spot strain.

[2]Normal commercial strain.

[3]Basal diet supplemented with 18 ppm copper.

[4]Basal low-copper diet.

any treatment differences. The fertility and hatchability are listed in Table 17 and 18, respectively. Again, as a result of the small number of birds used and their comparatively low rate of production, no significant differences were detected between treatments or were any trends indicated in the results obtained.

Results of the copper analyses on the livers are presented in Table 19. Treatment was significant at the 1% level. The copper levels in the livers of the 2 strains of birds fed the supplemented diet were almost identical. The mean level in the normal group was 21.16 ppm and in the blood-spot group was 21.22 ppm. The copper levels of the 2 groups fed the deficient diet were not only significantly lower ($P < 0.01$) than their respective controls but were significantly different ($P < 0.01$) from each other. The livers from the normal strain fed the basal diet contained 13 ppm of copper as compared to the livers from blood-spot birds which contained 16 ppm. These results clearly indicate that the normal strain was more sensitive to a dietary copper deficiency. The supplemental diet containing 18 ppm of copper was well above the supplemental level used by other workers (Hill and Matrone 1958, 1961). However, the 21 ppm of copper in the liver of the control birds is below the 32 ppm found in 3 livers from laying hens of the normal strain fed the standard type diet in

TABLE 17

Percent fertility of normal and blood-spot hens fed a copper-deficient diet

M	F	Diet	No. Birds	Averages for 3-day periods					
				1	2	3	4	5	6
BS	BS[1]	Cu^{+}[3]	5	100	85.7	100	80.0	80.0	100
N	N[2]	Cu^{+}	6	100	100	100	100	100	100
BS	BS	Cu^{-}[4]	5	80.0	100	100	87.5	71.4	87.5
N	N	Cu^{-}	7	100	100	100	100	100	100

[1]Blood-spot strain.

[2]Normal commercial strain.

[3]Basal diet supplemented with 18ppm copper.

[4]Basal low-copper diet.

TABLE 18

Percent hatchability of fertile eggs from normal and blood-spot hens fed a copper-deficient diet

M	F	Diet	No. Birds	Averages for 3-day periods 1	2	3	4	5	6
BS	BS[1]	Cu^{+}[3]	5	100	83.3	83.3	50.0	75.0	44.4
N	N[2]	Cu^{+}	6	100	90.9	80.0	100	80.0	81.3
BS	BS	Cu^{-}[4]	5	50.0	60.0	57.1	85.7	80.0	85.7
N	N	Cu^{-}	7	100	100	78.6	88.9	100	66.7

[1]Blood-spot strain.

[2]Normal commercial strain.

[3]Basal diet supplemented with 18 ppm copper.

[4]Basal low copper diet.

TABLE 19

Copper levels in the liver of laying hens fed a copper-deficient diet for 18 days

Strain		BS	N	BS	N
Diet		Cu supp.[1]	Cu supp.	Cu def.[2]	Cu def.
Liver number	1	23.46[3]	21.68	15.77	11.57
	2	23.89	17.45	17.19	9.35
	3	18.21	20.40	16.02	10.23
	4	19.69	25.04	22.14	13.80
	5	19.16	25.45	15.37	14.51
	6	27.67	18.89	13.84	22.27
	7	16.48	19.24	13.51	15.51
mean[4]		21.22[a]	21.16[a]	16.26[b]	13.89[b]
SEM		± 1.48	± 1.61	± 1.09	± 1.64

[1]Copper supplemented diet contained 18 ppm.

[2]Basal diet contained 3 ppm.

[3]Average of 2 determinations in ppm.

[4]Means with different superscripts are significantly different ($P < 0.01$).

this study. This decrease in the levels of liver copper follows the same pattern found in the rates of production. The normal strain in both cases was more sensitive to the low-copper diet.

Results of the iron analysis of these livers are presented in Table 20. The analysis of variance indicated that the 4 group means were significantly different at the 1% level. The iron level in both strains was significantly decreased by feeding the low-copper diet. The differences in the level of liver iron between the 2 groups fed the same diet were not significant. The normal hens showed the greater decrease in liver iron due to the dietary treatment, decreasing from the control level of 561 ppm when fed the supplemental diet to 308 ppm in the group fed the basal diet. This compares with the blood-spot strain which decreased from a level of 496 ppm iron in the controls to 377 ppm in the group fed the low-copper diet. This decrease in the level of liver iron as a result of feeding a low-copper diet is not unexpected. Maynard and Loosli (1969) state in their text that when copper is deficient in the diet of swine, there is a decreased absorption of iron, a lowering of its total body content and a decrease in its mobilization from the tissues.

These results clearly indicate that the hens fed the experimental diet were in a stress condition caused by

TABLE 20

Iron levels in the liver of laying hens fed a copper-deficient diet for 18 days

Strain		BS	N	BS	N
Diet		Cu supp.[1]	Cu supp.	Cu def.[2]	Cu def.
Liver number	1	381[3]	649	649	265
	2	351	540	374	317
	3	624	787	445	210
	4	522	525	259	335
	5	678	692	266	422
	6	610	367	286	312
	7	307	367	363	298
mean[4]		496.1[a]	561.0[a]	377.4[b]	308.4[b]
SEM		± 56.4	± 60.4	± 51.9	± 24.6

[1]Copper supplemented diet contained 18 ppm.

[2]Basal diet contained 3 ppm.

[3]Average of 2 determinations in ppm.

[4]Means with different superscript are significantly different ($P < 0.01$).

a copper deficiency. However, the observed effect of this dietary deficiency of a single nutritional element may ultimately result from its effect, in turn, upon the level and availability of other elements in the bird.

Except for 2 groups, all the samples were lost prior to sectioning. These aortas were removed from the chicks of hens which had been fed the supplemented diet. A total of 19 aortas were sectioned and examined for abnormalities using the electron microscope (Table 21). The examination of the aorta from a blood-spot chick from the fifth 3-day period, representing the thirteenth, fourteenth and fifteenth days the hen was fed the supplemental diet, was not conclusive. From this section, it could not be determined if there was a definite change in structure of the elastic fibers in the tunica of the artery. When the 6 aortas from the normal chicks of the same period were examined, 2 were normal and 2 were definitely altered. Alterations in the vascular connective tissue are generally characterized by fragmentation and disruption of the elastic fibers. Of the 4 aortas examined from the blood-spot strain taken from the sixth 3-day period, representing the sixteenth, seventeenth and eighteenth day on the supplemental control diet, all but one of the aortas appeared to be altered. Of the 8 aortas removed from normal chicks during the same period, 1 was normal and unchanged,

TABLE 21

Observations of electron micrographs of aortas from day old chicks treated with a low-copper parental diet

Strain	Parental[1] period	aorta examined							
		1	2	3	4	5	6	7	8
Blood-spot	5	+-							
Normal	5	+	+	+	+	-	-		
Blood-spot	6	+	+	+-	-				
Normal	6	+	+	+	+	+	+-	+-	+-

Note: (+)Abnormal, change in structure of aorta wall.

(+-)Possibly abnormal.

(-)No change in structure detected.

[1]Period of parental dietary treatment, diet contained 18 ppm copper.

2 were border-line and their condition not definitely determined, and the remaining 6 were definitely abnormal.

It is significant that such a large number of aortas from chicks whose parental diet contained 18 ppm copper possessed abnormalities characteristic of a copper deficiency. The National Research Council lists a requirement of only 6 ppm copper for the chick, and, for older birds, the requirement has not been determined.

Experiment IV

The 3 previous studies demonstrated that the observed reaction of the laying hens fed the experimental diet was a result of the stress condition induced by a copper deficiency. The effects of the copper deficiency reported in these studies were similar to those of Bird et al. (1963) and Simpson et al. (1967) but they were not identical in all respects. These authors reported only slight decreases in the production of treated birds. While the dietary effects of these studies are attributed entirely to a dietary copper deficiency, other factors must be involved which, if recognized, would account for the differences in the observed results. The genetic background of the birds used in conducting nutritional studies is recognized as a source of variation in the results obtained. Washburn (1969) found significant ($P < 0.01$) interactions

between genotype and the diet used when studying the hematological response of different stocks of chickens to iron-copper deficiencies. A comparison of the diet used by Simpson et al. (1967) and that used in the previous studies revealed that these 2 diets not only differed in the form in which some of the essential inorganic elements were added but also in the presence of magnesium and cobalt.

This study was conducted to determine the effect of differences in the mineral content of diets used in studying copper deficiency upon a normal strain of White Leghorns.

Procedure

The effect of 3 semisynthetic low-copper diets upon production, hatchability and fertility were compared (Table 22). The first diet was formulated according to Simpson et al. (1967). This was a low sulfate diet to which neither magnesium or cobalt salts were added. The second diet was identical to the first except for the addition of sodium sulfate. The addition of this salt increased the sulfate in the first diet to a level equal to that present in the third diet. The third diet was identical to that used in the previous studies which included salts of magnesium and cobalt. A practical type farm laying diet was used as a fourth treatment. It served

TABLE 22

Composition of semisynthetic low-copper layer diets[1]

Ingredients	% of Diet		
	1	2	3
Dried skim milk	50.0	50.0	50.0
Cerelose	35.0	35.0	32.0
Corn oil	5.0	5.0	5.0
Glycine	0.5	0.5	0.5
DL-methionine	0.3	0.3	0.3
Choline Cl (25%)	0.2	0.2	0.2
$CaCO_3$	5.0	5.0	6.0
NaH_2PO_4	. .	. .	4.0
$CaHPO_4$	2.0	2.0	. .
Microingredients[2]	1.0	1.0	1.0
Salts	**(grams/100 pounds)**		
NaCl	90.80	90.80	136.05
$ZnCO_3$	20.43	20.43	. .
$ZnCl_2$	. .	. .	0.50
Fe citrate	27.24	27.24	. .
$FeSO_4 \cdot 7H_2O$	. .	. .	27.00
$MnCO_3$	14.07	14.07	. .
$MnSO_4$	. .	. .	15.25
KIO_3	0.18	0.18	. .
KCl	. .	. .	205.50
$CoCl_2$	. .	. .	0.02
$MgSO_4$	. .	. .	136.05
$NaSO_4$	. .	200.00	. .

[1]By analysis contained 2.0ppm copper.

[2]Vitamins added per 100g of diet: vitamin A, 2000 USP units; vitamin D_3, 433 ICU; and (in milligrams) menadione, 2.5; α-tocopheryl acetate, 2.5; Santoquin, 0.0125; thiamine-HCl, 1.0; riboflavin, 1.0; pyridoxine-HCl, 1.0; Ca pantothenate, 3.0; niacin, 5.0; inositol, 50; biotin, 0.04; folic acid, 0.2; and vitamin B_{12}, 0.003.

as a positive control in determining the adaptability of the hens to the copper-deficient diets. Each of the 3 low-copper and the positive control diets were fed to 2 replicate groups of 6 birds each for a period of 15 days following the procedure outlined in the previous studies. The birds used in this study were a commercial strain of White Leghorns.

Results and Discussion

The analysis of variance showed that the 4 dietary treatments differed significantly in their effect upon the rate of production at the 0.5% level. The production of those birds receiving the unsupplemented and sulfate-supplemented diet of Simpson et al. (1967) during the experimental period was significantly lower than the production of the other groups. When the results of the 2 replicates were combined, the birds in the first group produced an average of 4.8 eggs during the 15-day experimental period. This production rate is only slightly higher than the average of 4.2 eggs produced by birds fed the same diet supplemented with sulfate. The difference between these groups was not significant. Those birds fed the experimental diet used in the previous studies produced an average of 7.3 eggs during the same period. All birds fed the low-copper diets produced at a significantly lower

($P < 0.01$) rate than the positive controls which produced an average of 12.4 eggs during the 15-day period.

The effects of the 4 dietary treatments upon both fertility and hatchability were significant at the 1% level when analyzed by chi-square. The unsupplemented and sulfate-supplemented diet of Simpson et al. (1967) resulted in the greatest incidence of infertile eggs produced as well as the lowest hatchability. While the effect of these 2 diets upon fertility and hatchability were the same, they were significantly different ($P < 0.01$) from the fertility and hatchability of the remaining 2 dietary treatments. The positive control hens exhibited the lowest incidence of infertility and the greatest hatchability. In both cases (fertility and hatchability), the results were significant at the 1% level when compared to the results obtained from those groups receiving the semisynthetic diets.

Bird et al. (1963) do not give the complete dietary formula used, reporting only that it was a nonfat milk-solids diet and that only a slight depression in egg production was observed after feeding the low-copper diet for 20 weeks. Simpson et al. (1967) reported that egg production, as a result of feeding a low-copper diet, was not significantly affected. These authors do report that a group receiving the semisynthetic diet supplemented with 46 ppm of copper produced at a slightly lower rate compared

to hens receiving a practical type diet. Both workers report a reduction in the hatchability of fertile eggs. Bird et al. (1963) observed a reduction in hatachability of fertile eggs from 83 to 11%. Simpson et al. (1967) stated that hatchability was reduced to zero after the birds had been fed the low-copper diet for 10 days.

One of the major differences between the experimental diet used in the previous studies and that used by Simpson et al. (1967) was the form in which some of the essential elements were added. In the diet of Simpson et al. (1967), only the manganese was added as the sulfate salt. In the low-copper diet of the previous studies, the manganese, as well as the iron, was added in the sulfate form. The nutritional role of sulfate has been the subject of many studies reported in the literature; good examples are those of Machlin (1955) and Button et al. (1965). Brown et al. (1965) reported that decreasing the level of dietary sulfate decreased the production of collagen by preventing the formation of cross-linkages. In 1967, Brown found that the extendability of aortas was decreased when rats were fed a diet low in sulfate.

In the present study, the sulfate level does not appear to be the dietary factor responsible for the difference in results. Morrison (1959) reports the need for magnesium

supplementation when feeding a diet of whole milk as well as for iron and copper. Magnesium was not added in the diet of Simpson et al. (1967) but was added to the diet used in the previous studies as the sulfate salt.

Summary

A series of experiments were conducted to determine if differences could be detected between the integrity of the vascular connective tissue of a normal and blood-spot strain of bird. These 2 strains were compared in stress and nonstress environments. The definable stress environment was created by feeding both a copper-deficient diet and a diet containing toxic levels of BAPN. Both these diets are known to exert their effect upon the vascular connective tissue, preventing the formation of normal collagen and elastin. With deficiencies present in the connective tissue of the blood-spot strain, it would be expected that they would be more susceptable to stress placed on the connective tissue.

The statistical analyses of the results showed significant differences between the response of the 2 strains to these dietary treatments. However, abnormalities in the vascular system of the blood-spot strain were not detected. The normal strain proved to be the more susceptable of the

two under the experimental conditions. The environmental stress resulting from the experimental dietary procedure did not produce any observable response in the blood-spot strain; such a response would have indicated an absence of vascular integrity (abnormal or deficient connective tissue).

Interpretation of the results must be made with 2 factors in mind. First, the difference between the 2 strains in their response to the dietary treatment may result entirely from a variation in their nutritional requirements and not be related in any way by factors contributing to the incidence in blood-spot production. Secondly, vascular abnormalities present in the follicular wall may not be indicative of the state of the vascular system throughout the body. If weaknesses in the connective tissue of the vascular system in the blood-spot strain exist, they are probably isolated in the follicle. Whatever deficiencies are present in the follicles of the blood-spot hens, these conditions do not appear to be characteristic of the entire vascular system.

A more direct approach is necessary if the primary factors responsible for the occurrence of blood spots is to be resolved. It is obvious that, when comparing the results of feeding semisynthetic diets, the interaction of elements present, especially when the diet is deficient in an essential element, must be taken into consideration.

CHAPTER III

RATE OF OVA GROWTH AND OVA SIZE IN BIRDS OF THE NORMAL AND BLOOD SPOT STRAIN

Introduction

In studying the occurrence of blood spots, the critical period is from the initiation of the rapid phase of ova growth until ovulation. During this period, hemorrhage may occur, ultimately resulting in the presence of a blood spot in the egg. Differences between the integrity of the follicular vascular system of the two strains of birds would not be the only abnormality which could account for a high incidence of blood spots. A more accelerated increase in the yolk size during the rapid phase of ova growth or a larger yolk at maturity could produce a stress upon the follicle wall resulting in ruptures of the blood vessels.

Stiles and Dawson (1959), when comparing normal and blood-spot eggs from a similar clutch position, found that the average oviposition time of eggs containing blood spots occurred 30 to 50 minutes sooner than normal eggs. They also reported that the average weight of eggs containing blood spots was significantly greater than the average weight of normal eggs when comparisons were made

between eggs of the first clutch position and when all the eggs were compared disregarding clutch position. The authors concluded that eggs containing blood spots were ovulated sooner, weighed more and that these were abnormal eggs at or prior to ovulation. Total egg weight is of little value in attempting to determine the condition which exists during the critical period, since the albumen which accounts for the majority of variation in egg size and the shell are formed after the formation of blood spots.

The period of rapid development of the ovum has been traced by using a fat-soluble dye incorporation technique in studies reported by Warren and Conrad (1939) and by Bacon and Skula (1968). After a thorough survey of the literature, no reference was found where either the rate of ova growth during the rapid phase or yolk size at maturity were studied in relation to the occurrence of blood spots.

Physiologically, interest in the problem of blood spots is centered around the extremely rapid rate of follicular growth and the attendant development of the necessary blood supply. The avian follicle is the fastest growing structure known, developing as it does, from a size of about 2 mm in diameter to full ovulatory dimensions of 32 mm in diameter during a period of 9 days (Sturkie, 1965). It was desirable, therefore, to determine if the growth

rate and yolk size at maturity were abnormal in hens producing a high incidence of blood-spot eggs.

Procedure

Based upon production records and a high incidence of large blood spots, 45 birds in the first year of production were selected. The same number of control hens of the Babcock strain were selected on the basis of high production.

A gelatin capsule containing approximately 30 mg Oil Red O dye was orally administered to each of the 90 birds. Beginning on the date of dye administration, eggs were collected daily for a period of two weeks, and individually marked to identify each hen. All eggs were hard cooked the day following collection and bisected by cutting along the long axis through the latebra. Two perpendicular measurements (to the nearest 0.5 mm) were averaged in determining the diameter of both the whole yolk and the dye ring. The percent of yolk development at the time of dye administration was determined by calculating the ratio of the dye to the yolk diameter. The average of these ratios for both groups were plotted each day to determine the rate of yolk growth and the length of the rapid phase of growth. Egg production and the mature yolk size were also determined during the experimental period for the hens in each group.

Results and Discussion

The rates of ova growth for the normal and blood-spot birds used in this study are plotted in Figure 2 with the variance for each day during the test period. The dye did not appear in the egg yolk until the third day after administration of the dye capsule. The data obtained failed to indicate any differences between eggs of the two strains in relation to ovarian function as measured in terms of rapid development period. In both strains, the ova required a period of about 8.5 days to reach maturity after initiation of the rapid phase of ova development. This period of rapid growth is indicated on the graph from the third to the ninth day of the experimental period. For the remainder of the experimental period, the size of the ova at time of dye administration remained constant, and results after the twelfth day are not shown. These two phases coincide with the periods of slow and rapid growth of the developing ova first reported by Warren and Conrad (1939).

Bacon and Skula (1968) noted that follicular maturation (cessation of rapid development) and ovulation were not necessarily simultaneous events. They report that differences exist in the length of the rest period, the time from the cessation of rapid growth until ovulation, and of ova in different clutch positions. From the data

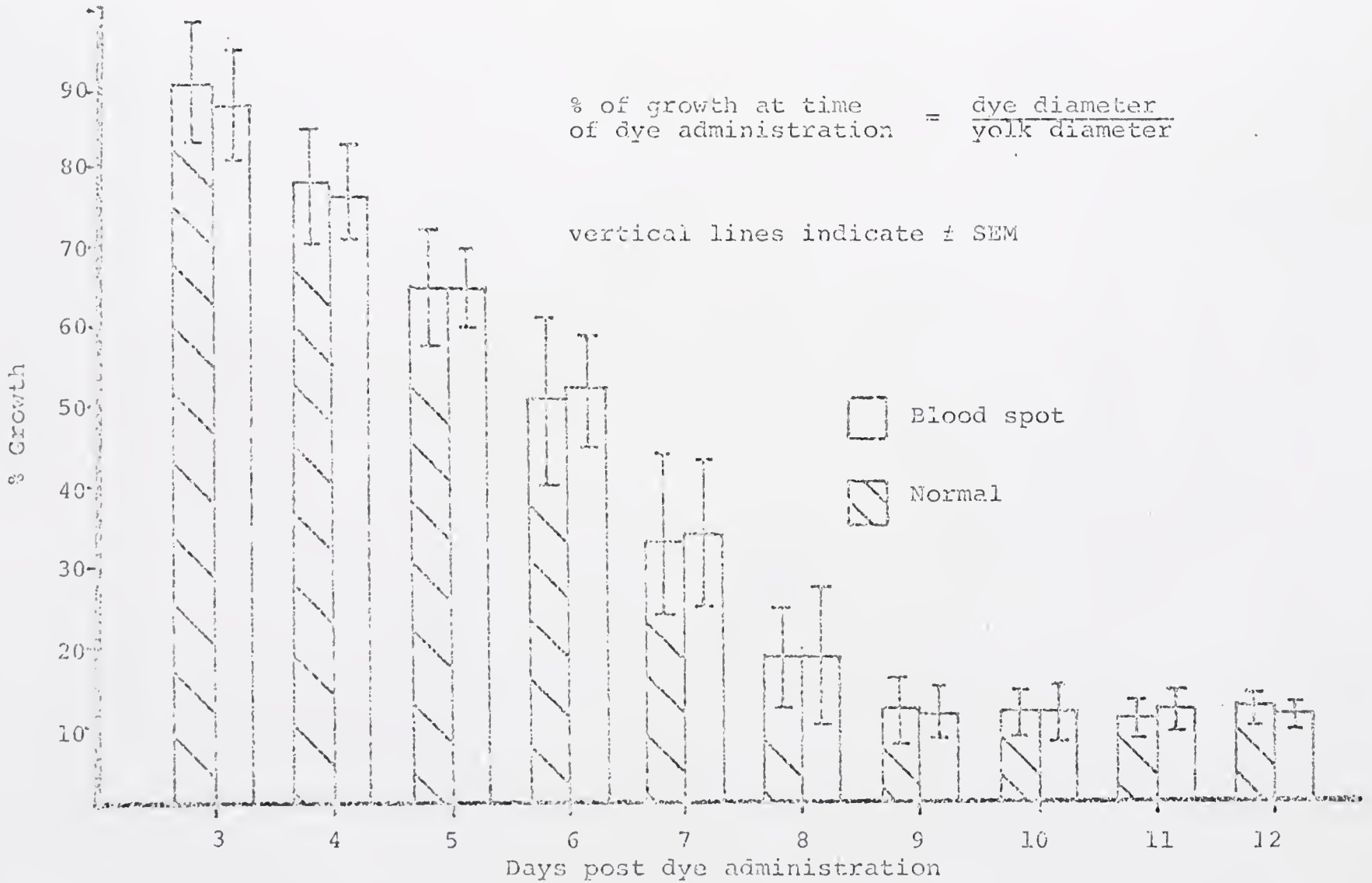

Fig. 2 Rate of yolk growth in N and BS strains.

obtained in this study, no difference in the rest period was detected between ova from the 2 strains, regardless of clutch position.

The average yolk size during the experimental period for each hen was treated as a unit and these units were statistically analyzed (Table 23). Yolks from the normal strain of hens were significantly larger ($P < 0.01$) than the yolks from the blood-spot strain of hen. It is possible that the relation of egg size and oviposition time to the incidence of blood spots reported by Stiles and Dawson (1959) is not a cause-and-effect relationship. Those factors responsible for blood-spot production may also result in a shorter oviposition time and larger egg size. Strain differences probably accounted for the larger ova size in the normal strain rather than in effects of factors which contribute to blood-spot incidence. These data, together with the length and rate of rapid growth, give no indication of differences between strains which could account for the difference in incidence of blood spots.

Summary

The rate of ova growth during the rapid phase of maturation and the ultimate size of the ova were compared in the normal and blood-spot strain. It is commonly accepted that the hemorrhage which results in the formation

TABLE 23

Average size of yolk produced by 42 hens of both the normal and blood-spot strain during a 10-day period

Blood Spot		Normal	
31.4[1]	32.4	31.5	31.6
31.9	32.1	30.8	32.9
31.3	31.0	30.0	30.8
30.9	31.4	31.0	32.1
29.1	31.2	32.0	30.8
31.3	31.7	33.4	32.6
31.9	31.5	31.8	31.7
30.6	30.0	31.3	33.3
31.8	30.8	33.2	31.8
32.2	30.7	32.6	31.7
31.8	32.6	31.2	32.2
30.8	31.0	32.0	32.8
31.6	31.1	32.4	31.2
30.4	31.5	31.9	32.2
31.4	32.2	32.1	31.3
31.4	31.8	32.9	33.3
29.9	31.6	30.7	31.1
31.3	31.7	32.9	31.4
30.8	32.0	31.1	31.3
32.9	33.4	31.1	31.9
30.6	31.9	32.7	34.0
mean ± SEM[2]	31.40 ± 0.12[a]		31.92 ± 0.14[b]

[1]Yolk diameter in mm.

[2]Mean yolk diameter, superscripts denote significant difference ($P < 0.01$).

of a blood spot may occur during the last few hours of rapid growth or just prior to ovulation. Stress sufficient to result in a hemorrhage could conceivably result from an excessive rate of ova growth or an abnormally large ovaum in the blood-spot strain. Using a dye technique, no detectable differences were observed between the rates of ova growth in the 2 strains of hens. In both strains, the length of the rapid growth phase of ova maturation was 8.5 days, which is within the expected range. While the mature ova of the normal strain were significantly larger ($P < 0.01$) than those of the blood-spot strain, this difference was quite small and does not appear to be related to the incidence of blood spots.

CHAPTER IV

THE RELATIONSHIP OF BLOOD PRESSURE TO THE INCIDENCE OF BLOOD SPOTS PRODUCED

Introduction

Blood pressure is often mentioned as a possible factor influencing the occurrence of blood spots. Nalbandov and Card (1944) reported a higher incidence of blood spots in birds having a higher blood pressure. This difference was not significant and the authors concluded that high arterial pressure is not the primary cause of rupture in follicular vessels. After comparing systolic blood pressure and blood-spot occurrence, Weiss (1958) concluded that the normal range of pressure in the White Leghorn does not materially influence the incidence of blood spots. In 1968, Fry et al., using a strain of White Leghorns that produced a large percent of blood-spot eggs, reported that blood pressure was significantly and positively correlated with both size and number of blood spots produced.

In this study, the effects of lowering the systolic pressure of laying hens upon the formation of blood spots was determined.

Procedure

Birds of the blood-spot strain were selected on the basis of a high incidence of large blood spots. Systolic pressures were indirectly measured by a physiograph-six (manufactured by E-M Instruments Co., Inc.) using a sphygmomanometer. The cuff was placed around the distal end of the tibia and the pneumatic pulse pick-up on the artery passing over the hock joint. Three determinations were recorded for each bird, and the average was used in subsequent calculations.

The systolic blood pressure was reduced using methimazole, a goitrogenic agent. The birds were treated by giving them free access to the standard farm laying diet containing 0.06% methimazole, commonly referred to as tapazole (registered trade-mark of Eli Lilly and Company for 1-methyl-2-mercaptoimidazole). The level of 0.06% was found, through a series of preliminary trials, to result in the greatest decrease in blood pressure with the least loss of production. When birds of the blood-spot strain were fed a level of 0.03% tapazole for an 18-day period, the systolic pressure was not significantly reduced. When the tapazole level was increased to 0.1% in the feed, the rate of production immediately decreased. After 11 days, all birds were out of production.

Initial systolic pressures were recorded and the tapazole-containing diet fed for a 30-day period, after which the birds were again fed the untreated diet for a 36-day post-treatment recovery period. During the entire 66-day experimental period, eggs were marked with the bird numbers and collected daily. They were broken out to determine the incidence of blood spots and the blood spots were scored according to the procedure previously described in the general procedure.

Results

The percent blood-spot eggs produced and the average blood-spot score were calculated for the individual hens during each 6-day period. The average systolic pressure with the standard error of the mean are indicated for the period in which they were recorded (Table 24). Significant differences in the average systolic pressure are indicated by the superscripts. The stress of handling the laying hens prohibited the recording of blood pressure during each 6-day period.

It can be seen that the feeding of tapazole significantly reduced the systolic pressure. Blood-spot scores were not significantly affected, although the average blood-spot score appeared to decrease and subsequently increase with the change in blood pressure. This may in

TABLE 24

The effect of decreasing the systolic pressure upon incidence and severity of blood-spots by feeding 0.06% tapazole to 16 birds of the blood-spot strain

	6-day periods										
	Dietary treatment (30 days)						Post-treatment (control diet)				
	1	2	3	4	5	6	7	8	9	10	11
Production	46.9	33.3	19.8	11.5	3.1	0	2.1	23.4	27.7	29.8	47.9
% BS	71.1	93.8	89.5	81.8	100	. .	100	36.4	50.0	67.9	66.7
BS score[1]											
(%) 1	9.4	13.3	29.4	11.1	0.0	. .	0.0	0.0	23.1	10.5	16.7
2	18.8	20.0	17.6	33.3	66.7	. .	50.0	37.5	30.8	31.6	23.3
3	12.5	16.7	11.8	0.0	0.0	. .	50.0	25.0	15.4	36.8	23.3
4	21.9	3.3	5.9	0.0	33.3	. .	0.0	12.5	0.0	0.0	10.0
5	34.4	46.7	35.3	55.6	0.0	. .	0.0	25.0	30.8	26.3	26.7
Mean score	3.44	3.50	3.00	3.56	2.67	. .	2.50	3.25	2.85	3.16	3.07
Systolic pressure[2]	151.1^{b} ±4.57		157.6^{ab} ±8.54		125.1^{c} ±5.14						173.7^{a} ±7.31

[1]Blood-spots were scored from 1 (smallest) to 5 (bloody albumen).

[2]Average systolic pressure with standard error of the mean.

part be due to the low production during the fifth and seventh 6-day periods. When the systolic pressures were recorded at the end of the dietary treatment, all birds ceased to produce for at least 7 days. It should be noted that at the end of the post-treatment period, the blood pressure in these birds had returned to a significantly higher level than was initially recorded. This increase in pressure was not accompanied by an increase in the percent or average score of blood spots produced. It was assumed that if systolic pressure was directly related to blood-spot production, the incidence and size of blood spots produced could be altered by significantly changing the blood pressure. Results show that when the systolic pressure was significantly decreased and subsequently increased, the incidence and score of blood spots were not similarly affected.

It must be remembered that while arterial pressure is assumed to be indicative of the capillary pressure, local hemorrhagic controls in the follicle may be active; therefore, the recorded pressure may not at all be indicative of blood pressures present in the follicle vessels.

It is concluded from the data in this study that if arterial pressure is related to the formation of blood spots, it has only a very minor role.

Summary

Since blood spots result from intrafollicular hemorrhage, blood pressure is often implicated as a causative factor in their formation. In this study, the systolic pressure in the blood-spot strain was significantly altered by feeding 0.6% tapazole, a goitrogenic agent. While the systolic blood pressure was significantly decreased and ultimately increased from the normal pretreatment pressure, no significant change in the incidence or size of blood spots were observed. While arterial and capillary pressure are generally correlated, they are not necessarily so, and hemostatic controls localized in the ovary and oviduct could, therefore, operate to maintain a uniform pressure in the follicle vessels.

CHAPTER V

CHARACTERIZATION OF FOLLICLES FROM HENS OF THE NORMAL AND BLOOD SPOT STRAIN

Introduction

No reports were found in the literature where the physical condition of the ovaries were examined for the presence of any abnormalities in hens known to lay eggs with a high incidence of blood spots. Shirley (1965) describes the occurrence of an intrafollicular hemorrhage which resulted in the subsequent formation of a blood spot in a laparotomized hen of a normal strain. The author observed no abnormality other than the rupture which resulted in the blood spot. The present study was conducted to determine if differences between the follicle of a normal and a blood-spot strain of hen could be visually detected.

Procedure

One-hundred and sixty-two birds of the normal strain and 51 birds of the blood-spot strain, both producing at a normal rate, were selected. The birds were exanguinated and the abdominal cavity opened. The three largest follicles present in the ovary of each bird were excised

with the stalk intact. Upon examination, the blood present in the stalk of each follicle was forced into the vessels of the wall. In this manner, the vessels surrounding the stigma were readily distinguishable. In comparing the follicles from the two strains of birds, the presence of several characteristics were enumerated. First, the stigma was observed and placed into one of two categories (branched or unbranched) according to its shape. If the stigma had a shape different from that of a straight line, it was termed branched. The stigma was further characterized by the manner in which the large blood vessels were oriented in the surrounding area. If the stigma was outlined by the presence of large parallel vessels on each side, it was denoted as being well defined. If, on the other hand, the many large vessels surrounding the stigma, approached it and disappeared before crossing, it was listed as being not well defined. The follicles were closely examined to detect the presence of any hemorrhages in the wall. The width of the stigma was observed and any which exceeded 1 cm were recorded.

Results

The results of these observations are shown in Table 25. The occurrence of branched stigmas was almost three times higher in the blood-spot than in the normal strain.

TABLE 25

Comparison of ova characteristics in hens of the normal and blood-spot strain

Characteristics	(%)	
Strain	Normal	Blood-spot
Branched stigma (y shaped)	4.9	11.8
Well-defined stigma[1]	6.2	17.0
Stigma not well-defined	93.8	83.0
Blood clots in follicle wall	5.4	26.0
Wide stigma (>1cm)	0.0	9.8

[1]Well-defined stigma were characterized by a clearly differentiated border outlining the avascular stigma. They were often bordered by a large blood vessel which ran parallel to the stigma.

It is not known if any relationship exists between the incidence of branched stigmas and blood spots. It is concluded that, while branched stigmas are uncommon, they should not be termed as being abnormal.

A larger number of well defined stigmas (17%) were observed in the follicles of the blood-spot strain than in the normal strain (6%). The manner in which the blood vessels are oriented in differentiating the stigmas from the rest of the follicle wall can not be directly related to the occurrence of blood spots.

The blood found in the follicle walls of both strains of birds appeared to have resulted from ruptured vessels. This blood appeared to be between the theca interna and theca externa and not adjacent to the vitelline membrane of the ovum. These areas of blood were approximately 1 cm in diameter and were easily detected. They were present before the follicles were excised and did not result from handling. They were observed in 26% of the follicles from the blood-spot strain as compared to only 5% found in those from the normal strain. It was often observed that a follicle contained more than one bloody area in the wall.

While no wide stigma were observed in the follicles of normal birds, 10% of those from the blood-spot strain were classified as wide. These wide stigmas were not only

present in the largest follicle of a hen but also in those not yet mature. These last two characteristics could possibly be related to the occurrence of blood spots.

Ovulation has frequently been observed but the mechanism by which it occurs remains unknown. Rupture of the follicle takes place at the stigma but the immediate cause has not been elucidated. Nalbandov (1964) states that the follicle "blanches" because of the drastically decreased blood flow through it. The stigma then becomes wider and many of the capillaries that extend across it become constricted and devoid of blood. This theory would not explain the appearance of wide stigma in small maturing follicles.

Summary

The literature reviewed contained no reports of examinations of follicles from birds producing an excessive number of blood-spot eggs. In this study, the three largest follicles from layers of both the normal and blood-spot strain were removed and examined. The observation of specific physical characteristics were enumerated for the purpose of making a comparison of the two strains. Two striking differences were noted. First, a higher incidence of blood clots were observed in the follicle wall in the blood-spot strain. This incidence was more than 25% in the

blood-spot strain as compared with 5% in the normal strain. Secondly, almost 10% of the follicles from the blood-spot hens had unusually wide stigmas (> 1 cm). These wide stigmas were found not only in the largest follicles but also in the smaller, less mature ones. At this time, these differences in follicular characteristics can not be related to the difference in incidence of blood-spot eggs.

CHAPTER VI

GENERAL DISCUSSION AND SUMMARY

It is conservatively estimated that the egg industry loses over one million dollars each year from the occurrence of blood spots in eggs. The first extensive investigations dealing with this problem were reported in the early 1940's. Since that time, studies relating many factors with the incidence of blood spots have been published. It is the accepted theory that blood spots result from intrafollicular hemorrhages occurring within a nine-hour period prior to ovulation. However, the primary factor responsible for the rupture and subsequent formation of a blood spot has not been established. Possible causes for the loss of blood from ruptured vessels in the follicle wall include abnormally weak connective tissue in the vessel or follicle walls, abnormal maturation of the follicle and abnormally high blood pressure.

A major difficulty in studying this problem occurs when the hens are handled and a significant decrease or total loss of production results. It was this fact that necessitated the development of the dietary method used in these studies. For the purpose of investigating this problem, a strain of bird producing eggs with a high

incidence of blood spots was compared with a normal, commercial strain.

The first series of studies were conducted to determine if differences could be detected between the integrity of the vascular connective tissue of the normal and blood-spot strain. The progeny from hens of both strains, treated and untreated with the low-copper parental diet, were subjected to the stress of BAPN toxicity. Both a dietary copper deficiency and BAPN toxicity are known to affect the integrity of vascular connective tissue. The effects of the copper deficiency and BAPN toxicity were determined; additionally the response of chicks pretreated with the parental low-copper diet to BAPN toxicity was shown. If the high incidence of blood spots produced by the experimental strain results from abnormal or deficient connective tissue, then it would be expected that these birds would be predisposed to the experimental stress conditions.

Results indicated that the normal birds were the more susceptible of the 2 strains. Production decreased from 80% to less than 6% in five days. During the same period, production in the blood-spot strain showed only a slight decrease. The level of copper in the livers of the normal hens was reduced to a significantly lower level than in the blood-spot strain by the low-copper

dietary treatment. These observations, however, cannot be related to the condition of connective tissue and are probably the result of differences in nutritional requirements.

Hemorrhages which result in the formation of blood spots occur during the rapid phase of ova maturation. During the last phase of ova growth, the rapid phase, the follicle increases in size from about 2 mm to 32 mm in diameter in a 9-day period. The rates of growth during the rapid phase were compared in the 2 strains, using a dye technique. If the blood-spot strain matured at an excessive rate, this would be an indication that the follicles in these birds were under additional stress which could be directly related to the occurrence of vascular ruptures. The results did not indicate any difference in the rate of ova growth during maturation between the 2 strains. Furthermore, the size of the mature ova were found to be significantly smaller in the blood-spot strain. It is concluded that neither the rate of growth nor ova size are responsible for the high incidence of blood spots.

The blood pressure in the blood-spot strain was significantly affected by the feeding of tapazole. The results indicated that neither the significant increase or decrease in the systolic pressure resulted in a change in the percent or size of blood spots produced. It is

believed that if the systolic pressure were directly involved in blood-spot formation, then any change in pressure would result in obvious changes in the blood-spot incidence.

In the final experiment, the three largest follicles from hens of both strains were excised and examined to determine their physical characteristics. Observations were made comparing the manner in which the stigma were defined, whether the stigma were branched or unbranched, the width of the stigma and the presence of blood clots in the follicle wall. It should be noted that blood clots were found in the follicle walls of both strains; these appeared to have resulted from vessels ruptured during the rapid growth phase. The data from these studies and those reported in the literature do not, in any way, indicate that blood spots occur as a result of a physical abnormality in the follicle. It is evident from the presence of blood clots observed in the follicle wall of both strains that ruptures of follicular vessels are not uncommon. If blood flow in the follicle were not diminished prior to ovulation, then conditions would be present which could explain the large numbers of blood-spot eggs produced by the experimental strain.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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